The Effect of Glutamine on Rat Skeletal Muscle Composition Following Acute Spinal Cord Injury

A thesis submitted to the College of Graduate Studies and Research

in partial fulfillment of the requirements for the

Degree of Master of Science

in the Department of Anatomy and Cell Biology,

University of Saskatchewan, Saskatoon, Canada.

Prepared by Jamie Danielle Golding

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Abstract

Primary spinal cord injury (SCI) results from direct mechanical damage to the spinal cord. The resulting pathochemical and pathophysiological events, including oxidative stress and inflammation, lead to secondary injury. The ability to decrease secondary injury may lead to improved recovery. Increasing glutathione production after SCI leads to decreased secondary injury. Glutamine is an important precursor to glutathione following trauma. Skeletal muscle phenotype is strongly influenced by neuromuscular activity. SCI causes myosin heavy chain (MyHC) profiles to shift towards faster isoforms in slow muscles and slower isoforms in fast muscles. The hypothesis was that glutamine, as a precursor of glutathione, administration to SCI rats would lead to better functional recovery and a more preserved MyHC phenotype in locomotory muscles.

Rats were assigned to one of four groups; healthy, laminectomy only, untreated SCI, and SCI treated with an intraperitoneal injection of 1mmol/kg glutamine every 12 hours for one week after injury. SCIs were perfomed at T6 with a modified aneurism clip. Functional recovery was measured weekly using the Basso-Beattie-Bresnahan scale and the angle board method. Six weeks later, all rats were killed, and their extensor digitorum longus and soleus muscles excised and weighed. MyHC composition of the muscles was determined using SDS-PAGE.

The hypothesis that glutamine treatment following SCI would lead to better functional recovery and a more preserved MyHC profile was validated. Glutamine treated rats received significantly higher BBB scores ($p<0.01$) and angle board scores ($p<0.001$) than untreated SCI rats. Glutamine treatment also reduces muscle atrophy in

the soleus muscle, but not the extensor digitorum longus (EDL). In untreated rats the soleus muscle accounted for significantly ($p<0.001$) less of the percentage of total body weight than the soleus muscle from glutamine treated rats. Finally, SCI rats with preserved functional abilities displayed a significantly better preserved MyHC profile compared to untreated SCI rats. In the soleus healthy rats contain 94% type 1 myosin, treated rats maintained 68% which was significantly ($p<0.001$) greater than 28% maintained by untreated rats. In the EDL healthy rats contain 55% type 2b myosin, treated rats maintained 32% which was greater than 26% type 2b myosin maintained by untreated rats.

Acknowledgements

I would like to thank my supervisor Dr. Ben Rosser for all of his help, direction, guidance, and support over the last few years. I am grateful for his patience during all of our meetings and for reading over the many drafts of this project. I would also like to thank Dr. Bernie Juurlink for his insight and help in putting my project together. Sarah Rigley was an important member of the team doing the spinal cord surgeries and helping me with behavioral testing and bladder expressions. She also offered useful input, stimulating discussions, and candy to keep me going.

I would like to thank the following people as well; Dr. Doug Syme from the University of Calgary, Dr. Mary Pato from the Department of Biochemistry, Mehri Herman, and Anita Givins. Dr. Syme provided the protocol for MyHC purification and gel electrophoresis. It was truly invaluable. Dr. Pato generously lent electrophoresis equipment. Mehri looked after the rats and helped with behavioral testing, and Anita provided some technical assistance. Committee members Dr. Pat Krone and Dr. Ron Doucette provided thoughtful ideas and advice along the way.

Finally I want to thank all of my friends who went for coffee, provided moral support, made me laugh, and kept me sane through it all! My family has remained behind me with their continual love, support, and encouragement through all my ups and downs. My cats Snooker and Spatzly, woke me up early every morning with their antics ensuring that I rarely slept in.

Funding was provided by a Canadian Institutes of Health Research grant awarded to Dr. Juurlink and Dr. Rosser, the Department of Anatomy and Cell Biology, and the College of Medicine.

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List of Abbreviations

º	Degrees
ºC	Degrees Celsius
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Basso-Beattie-Bresnahan
Ca^{+2}	Calcium
EDL	Extensor digitorum longus
g	Gram
GSH	Reduced glutathione
GSSG	Oxidized glutathione
kg	Kilogram
mg	milligram
mm	millimeter
mmol	Millimole
MyHC	Myosin heavy chain
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NS	Not significant
Pi	Inorganic orthophosphate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Rotations per minute
SCI	Spinal cord injury
SD	Standard deviation
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
V	Volts

1. Literature Review

1.1 Skeletal Muscle Composition

Skeletal muscle is a heterogeneous tissue that is highly organized to produce movement and force (Lutz and Lieber, 1999; Pette and Staron, 2000). Each muscle is composed of bundles of fascicles, each containing numerous multinucleated cells called muscle fibers. Every muscle fiber contains numerous protein filaments known as myofibrils, which are the contractile elements of skeletal muscle. Contractile segments of myofibrils, termed sarcomeres, are composed of two types of myofilaments. Thin filaments are mainly composed of actin molecules and thick filaments are made up of myosin molecules. It is the movement of the thick and thin filaments in relation to one another that produce contraction (Sciote and Morris, 2000).

1.1.1 Classification of skeletal muscle fiber types

Skeletal muscle is composed of different fiber types with varying anatomical, molecular, metabolic, structural, and contractile properties (Pette and Vrbova, 1985; Pette and Staron, 2001; Pette, 2002). Discovery of these characteristics led to a progression of various classification schemes. One of the first classification schemes for mammalian skeletal muscle was based on fiber colors (Spangenburg and Booth, 2003). In 1873, due to their anatomical color and response to electrical stimulation, Ranvier first described slow contracting muscles as ‘red’ and fast contracting muscles as ‘white’. Later research found that not all red muscles were slow contracting, and expanded the descriptions of red and white muscle fibers also including a category of intermediate

fibers (Close, 1972). This led to the development of classification schemes based on fiber contraction speed, and oxidative capacity (Spangenburg and Booth, 2003). Fast contracting fibers have few mitochondria, low myoglobin content, high glycolytic and low oxidative metabolism, and are highly fatigable. Conversely, slow contracting fibers have many mitochondria, a high content of myoglobin, high oxidative metabolism, and fatigue slowly. Intermediate fibers fall in between white and red fibers in their properties (Close, 1972).

Further studies found that in any given fiber the thick filaments contained the same myosin isoforms, and had a high correlation to histochemically defined fiber types (Termin et al., 1989; Hughes et al., 1993; Pette and Staron, 1997; Talmadge, 2000). Myosin is a molecule composed of light and heavy subunits. Mammalian skeletal muscle contains four predominant myosin heavy chain (MyHC) isoforms, or proteins with similar functions and amino acid sequences, that determine the force-velocity characteristics of a fiber (Staron and Pette, 1986; Sciote and Morris, 2000) and the kinetics of its stretch-activation (Pette and Staron, 2001).

The current classification scheme is based on the MyHC isoforms present in muscle fibers (Schiaffino and Reggiani, 1996; Sciote and Morris, 2000) and is described as the most useful method to date (Pette and Staron, 1997; Pette et al., 1999; Pette and Staron, 2000, 2001; Spangenburg and Booth, 2003). Using this classification scheme, there are two main types of skeletal muscle fibers that are innervated by separate motor neurons. They are described as fast and slow fibers (Hughes and Salinas, 1999) based on their force-velocity relation (Schiaffino and Reggiani, 1996) and ATPase activities (Hamalainen and Pette, 1995). Fast fibers can be further broken down into three groups based on the MyHC isoforms that they express. Type IIB fibers contain type IIb MyHC

and are the fastest contracting fibers. They have few mitochondria, use glycolytic metabolism, and have a low resistance to fatigue. Type IIA fibers contain type IIa MyHC and are the slowest contracting of the fast fibers. These fibers have the highest oxidative capacity of the fast fibers and are relatively fatigue resistant. In between these two fiber types are type IIX fibers, containing IIx MyHC and having an intermediate contraction speed. The slow fibers, type I fibers, express type I MyHC and are the slowest contracting of all the fibers. Type I fibers use oxidative metabolism and are fatigue resistant (Pette, 2001; Pette and Staron, 2001; Pette, 2002; Spangenburg and Booth, 2003). Each of these four 'pure' fiber types express only one isoform of MyHC (Pette et al., 1999; Staron et al., 1999; Bobinac et al., 2000; Pette and Staron, 2000; Talmadge, 2000; Pette and Staron, 2001) and are found in muscles with specific functions. For example, muscles containing mainly type I fibers are used for an antigravity function and sustained locomotor activity. Muscles containing mainly type IIX and IIB fibers are specialized for short-duration, high output power activities such as sprinting or weightlifting, and IIA fibers are specialized for continuous contractions such as during long distance running (Jakubiec-Puka et al., 1999; Baldwin and Haddad, 2002). Nonetheless, it has been proposed that a new fiber type classification system may soon be determined according to genomic nomenclature (Spangenburg and Booth, 2003).

1.1.2 Myosin

Myosins form a superfamily of proteins with structurally similar head domains, but structurally distinct tail domains. The first classification scheme grouped myosins into two classes based on whether they contained one or two head domains. However,

as new myosins were identified, myosins were classified into seven distinct groups based on their structurally distinct tail domain structures (Cheney et al., 1993). Sarcomeric myosins are categorized as class II myosins (Cheney et al., 1993; Moss et al., 1995; Schiaffino and Reggiani, 1996) and contain two heads and a long tail. There are six other major classes of myosin proteins all containing one head and structurally distinct tail domain structures (Cheney et al., 1993).

Sarcomeric myosins are hexomeric molecules composed of two heavy chains, each containing a head and a tail, and two essential (alkali) light chains, and two regulatory (phosphorylatable) light chains (Schiaffino and Reggiani, 1994; Moss et al., 1995; Schiaffino and Reggiani, 1996; Pette and Staron, 1997; Weiss et al., 1999; Pette and Staron, 2000; Pette, 2002). Barany (1967) was the first to show that contractile properties were correlated with myosin isoforms and were a property of ATPase activity. Since then, others have shown that MyHCs determine ATPase activity (Staron and Pette, 1986; Fauteck and Kandarian, 1995; Weiss et al., 1999) and are correlated with velocity of shortening (Reiser et al., 1985; Schiaffino et al., 1989; Moss et al., 1995; Jakubiec-Puka et al., 1999; Pette and Staron, 2000; Talmadge, 2000; Pette and Staron, 2001). Although both MyHCs and myosin light chains both contribute to the maximum velocity of shortening, the alkali myosin light chain isoforms do not contribute to ATP consumption or tension development (Schiaffino and Reggiani, 1994; Pette and Staron, 1997). Therefore, it is the MyHCs that are responsible for the major functional differences between myosin isoforms (Weiss et al., 1999) and that determine skeletal muscle properties and fiber types (Bandman, 1999; Pette and Staron, 2000; Talmadge, 2000).

1.1.3 Mammalian myosin heavy chain isoforms

There are four major MyHC isoforms in the trunk and limb muscles of mature, small mammals. Other skeletal MyHCs exist, but are limited to specific muscles or are minimally expressed (Talmadge, 2000). The slow isoform is MyHCs I (β), and the three fast isoforms are know as MyHCs IIa, IIx/d, and IIb (Schiaffino et al., 1989; Schiaffino and Reggiani, 1994, 1996; Pette and Staron, 1997; Windisch et al., 1998; Weiss et al., 1999; Sciote and Morris, 2000; Baldwin and Haddad, 2002; Pette, 2002). MyHC I or Iβ represent the same MyHC isoform in different locations. MyHC I is present in skeletal muscle and MyHC Iβ is present in cardiac muscle (Sciote and Morris, 2000). Likewise, MyHC IIx and IId correspond to the same myosin isoform (Schiaffino and Reggiani, 1994), and will be referred to MyHC IIx in the following. The maximum contractile velocities of these isoforms are I<IIa<IIx<IIb (Talmadge, 2000). For a review of the major MyHCs and myosin light chains found in rat skeletal muscle see Shiaffino and Reggiani (1994).

1.2 Skeletal muscle adaptability

Skeletal muscle fibers are dynamic with an astonishing adaptive ability (Moss et al., 1995; Bobinac et al., 2000; Pette, 2001; Pette and Staron, 2001). They can adjust their molecular, functional, and metabolic characteristics in response to various factors including development, innervation, increased or decreased neuromuscular activity, overloading or unloading, hormones, and aging (Pette and Staron, 1997, 2001; Pette, 2002; Fluck and Hoppeler, 2003). While factors increasing neuromuscular activity or loading cause an increase in expression of slower protein isoforms and slow fibers, factors that decrease neuromuscular activity or loading generally cause increased

expression of faster protein isoforms and fiber types. However, these changes are influenced by the species involved, the fiber type, and the properties of the specific muscle (Pette and Staron, 1997).

Fiber type transitions are not random, but follow an orderly, sequential pattern of MyHC isoform transitions from I→ IIa→ IIx→ IIb or vice versa (Pette and Staron, 1990, 1997, 2000; Pette, 2001; Pette and Staron, 2001). Hybrid fibers, or fibers containing more than one MyHC isoform, often appear in transforming fibers and appear to bridge the gap between pure fiber types (Pette, 2001). The exact molecular elements and mechanisms of fiber type transitions are still unclear.

1.2.1 Neuron muscle interaction

A single motoneuron and the muscle fibers that it supplies is referred to as a motor unit (Pette and Vrbova, 1985; Roy et al., 1991). Sherrington (1894) was the first to ascertain that skeletal muscle fibers were organized as motor units. All of the fibers within a motor unit show similar biochemical and histochemical properties (Pette and Vrbova, 1985; Schiaffino et al., 1989; Sciote and Morris, 2000; Edgerton et al., 2002). Type 1 fibers are controlled by postural motoneurons, while type 2 fibers are controlled by phasic motoneurons (Pette and Vrbova, 1985). Thus, skeletal muscle heterogeneity is defined by functionally distinct motor units.

Neuromuscular activity is defined as the combination of electrical activity and mechanical loading that a muscle receives (Talmadge, 2000; Edgerton et al., 2002). It is widely agreed upon that neuromuscular activity or motor innervation is one of the most important factors for determining skeletal muscle phenotype, protein expression, and contractile properties (Buller et al., 1960; Buller et al., 1969; Samaha et al., 1970; Pette

and Vrbova, 1985; Pette and Staron, 1997; Gundersen, 1998; Jakubiec-Puka et al., 1999; Pette and Staron, 2000; Fluck and Hoppeler, 2003). The importance of the nerve in determining skeletal muscle properties and MyHC phenotype has been shown by cross-reinnervation studies, electrical innervation studies and denervation experiments (Esser et al., 1993; Huey and Bodine, 1998).

Cross-reinnervation experiments have shown that neuronal activity can change muscle fiber phenotypes and MyHC expression (Bandman, 1999). A slow muscle reinnervated by a fast nerve results in the slow muscle becoming faster and a fast muscle reinnervated by a slow nerve results in the fast muscle becoming slower (Buller et al., 1960; Guth, 1968; Buller et al., 1969; Samaha et al., 1970; Pette and Vrbova, 1985; Pette and Staron, 1997, 2000). Similarly, electrical stimulation experiments, both low and high frequency, cause changes in muscle phenotype via firing frequency and activation pattern (Pette and Staron, 1997, 2000; Fluck and Hoppeler, 2003). Denervation studies have shown that a lack of innervation causes fast muscles to become slower and slow muscles to become faster (Pette and Staron, 2000).

1.2.2 Decreased neuromuscular activity and mechanical unloading

There are several different models of decreased neuromuscular activity and mechanical loading including denervation, spinalization, immobilization, tenotomy, microgravity, and hindlimb suspension. Nonetheless, all of these models have similar outcomes (Pette and Staron, 1997, 2000; Pette, 2002). Models of reduced neural input cause slow muscles to become faster and fast muscles to become slower. For example, in the soleus, a slow muscle, there is a decreased expression of MyHC I, and increased expression of the fast MyHC isoforms (Jakubiec-Puka et al., 1990; Roy et al., 1991;

Pette and Staron, 1997; Huey and Bodine, 1998; Talmadge, 2000; Pette and Staron, 2001). In the extensor digitorum longus (EDL), a fast muscle, there is increased expression of slower MyHC isoforms and decreased expression of the faster MyHC isoforms (Jakubiec-Puka et al., 1999; Bobinac et al., 2000).

1.3 Spinal Cord Injury

In Canada, there are approximately 1,050 people who receive spinal cord injuries (SCI) every year. Currently about 40,000 individuals are living with a SCI (http://www.rickhansen.com/Foundation/foundation_scilinks.htm). SCIs are dynamic and undergo continuous changes during their first few hours, days, and even weeks after the injury. Spinal cord lesions increase in size over four weeks, although within three days the lesion can be divided into an area of tissue destruction with an area of edema surrounding it (Zhang et al., 1997). SCI has been determined to take place in two phases. A primary phase of mechanical injury followed by a secondary phase of further damage caused by the mechanical injury (reviewed by Tator and Fehlings, 1991; Sekhon and Fehlings, 2001; Dumont et al., 2001).

1.3.1 Primary Injury

Primary SCI is the mechanical trauma to the spinal cord resulting in tissue necrosis and loss of function (Sandler and Tator, 1976; Anderson and Hall, 1993). Mechanisms of primary injury may include impact with continuing compression, impact with transient compression only, distraction, or laceration/transection (Dumont et al., 2001). Primary injury principally damages the central grey matter and causes cell death at the site of the lesion, but does little harm to the surrounding white matter (reviewed in

Dumont et al., 2001a; Profyris et al., 2004). However, following the primary injury specific molecular and cellular events take place within minutes and continue for days to weeks. These events lead to white matter damage and the spread of injury and are called secondary mechanisms (reviewed in Schwab and Bartholdi, 1996; Dumont et al., 2001a; Tator and Koyanagi, 1997).

1.3.2 Secondary Injury

Secondary injury occurs in response to the primary injury and leads to further damage and additional cell death (reviewed in Dusart and Schwab, 1994; Hutchinson et al., 2001; Sekhon and Fehlings, 2001; Schultke et al., 2003). The extent of secondary injury determines the outcome of the injury (Dumont et al., 2001a). Various pathochemical and pathophysiological mechanisms contribute to secondary injury (Anderson and Hall, 1993). These include hemorrhage, ischemia, edema, axonal and neuronal necrosis, phospholipid hydrolysis, free radical formation, excitotoxins, inflammation, demyelination, cyst formation, and scar tissue development (reviewed in Tator and Fehlings, 1991; Schwab and Bartholdi, 1996; Profyris et al., 2004). Of these mechanisms glutamate excitotoxicity, oxidative stress, and inflammation play a major role in secondary processes.

1.3.2.1 Excitotoxicity

A major secondary mechanism is disruption in blood flow. Hemorrhage, vasoconstriction and a disruption of venous drainage all contribute (Sandler and Tator, 1976; see Tator, 1995; Schwab and Bartholdi, 1996; Juurlink and Paterson, 1998; Dumont et al., 2001a). A disruption in blood flow leads to ischemia, which causes

decreased ATP, and depolarization of cell membranes. These events lead to a massive release of glutamate, one of the excitatory neurotransmitters of the central nervous system, which accumulates producing direct damage to the spinal cord. Additionally, released glutamate binds to ionotropic receptors leading to calcium and sodium influx into the cell. Calcium overload activates enzymes leading to an increase in free radical production and oxidative stress (reviewed in Azbill et al., 1997; Juurlink and Paterson, 1998; Dumont et al., 2001a).

1.3.2.2. Oxidative stress

Oxidative stress is a condition in which strong oxidant production outweighs a cells scavenging capabilities. Oxidative stress is often associated with inflammation and tissue damage, and has been implicated in various clinical conditions (Christman et al., 1998; Christman et al., 2000; Juurlink, 2001). There is general agreement that oxidative stress is a central factor in the secondary injury process and that it leads to increased damage to the brain and spinal cord following trauma (reviewed in Juurlink and Paterson, 1998; Dumont et al., 2001a).

The production of free radicals such as reactive oxygen and nitrogen species (ROS and RNS respectively) are harmful because they contain an unpaired electron in the outer orbital which makes the compound unstable. The free electrons enable the reactive species to bind to lipids, proteins, DNA, and RNA causing cell injury and tissue dysfunction (Azbill et al., 1997; Lovat and Preiser, 2003). Common reactive species include superoxide, hydroxyl radical, nitric oxide, singlet oxygen, hydrogen peroxide, peroxynitrite, and hypochlorite (reviewed in Azbill et al., 1997; Juurlink and Paterson, 1998). Under normal conditions the cell contains defenses to effectively scavenge

reactive species; however, if oxidative stress overwhelms the defense networks then cellular damage and cell death results (reviewed in Valencia et al., 2002).
Oxidative stress is also deleterious because it feeds into a cycle promoting glutamate excitotoxicity, rises in intracellular Ca^{2+}, ATP depletion, and a further increase in oxidative stress (Juurlink and Paterson, 1998). Additionally, oxidative stress is thought to be harmful as it activates proinflammatory genes leading to inflammation and increased functional losses (Bethea et al., 1998; reviewed in Profyris et al., 2004). It is widely agreed upon that interventions should be aimed at reducing secondary injury in order to obtain improved functional outcome.

1.4 The role of glutamine and glutathione

1.4.1 Glutamine

Glutamine (Figure 1) is the most abundant free amino acid in the blood stream and in the body (see Lacey and Wilmore, 1990; Calder and Newsholme, 2002; Mates et al., 2002; Roth et al., 2002; Valencia et al., 2002). It has a molecular weight of 146.15 and is composed of 41.09% carbon, 32.84% oxygen, 19.17% nitrogen, and 6.90% hydrogen. Glutamine contains two amines, an α-amino group, and a terminal amide group. Glutamine has many diverse functions in humans, animals, and cultured cells (reviewed in Lacey and Wilmore, 1990). These include fuel and essential metabolic precursor for rapidly dividing cells (Newsholme, 2001; Watford, 2001; Wilmore, 2001; Mates et al., 2002; Tapiero et al., 2002; Oehler and Roth, 2003), carbon metabolism (Tapiero et al., 2002), protein synthesis (Mates et al., 2002; Tapiero et al., 2002), glucose homeostasis (Tapiero et al., 2002), and glutathione homeostasis (Watford, 2001; Mates et al., 2002; Tapiero et al., 2002).

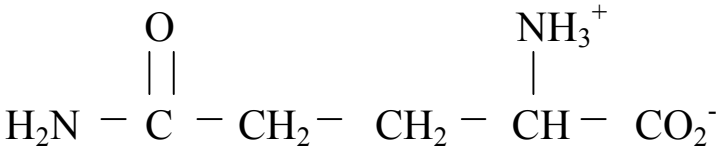


Figure 1. Structure of L-Glutamine (modified from Calder and Newholme, 2002).

Under normal circumstances glutamine is considered to be a nonessential amino acid (Calder and Newsholme, 2002). However, under certain circumstances where its consumption exceeds its synthesis, it is thought to become a conditionally essential amino acid (Amores-Sanchez and Medina, 1999; Newsholme, 2001; Watford, 2001; Tapiero et al., 2002; Kelly and Wischmeyer, 2003; Preiser and Wernerman, 2003). Such situations include major trauma or surgery, sepsis, bone marrow transplantation, intense chemotherapy or radiotherapy (reviewed in Valencia et al., 2001; Tapiero et al., 2002; Kelly and Wischmeyer, 2003). This observation has lead to the clinical use of glutamine in critically ill and post operative patients (reviewed in Lacey and Wilmore, 1990). Various studies have shown a beneficial effect of glutamine administration in improving long term survival and outcome (Lacey and Wilmore, 1990; Amores-Sanchez and Medina, 1999; Newsholme, 2001; Wernerman, 2003), decreasing hospital stay (reviewed in Wilmore, 2001), and attenuating oxidative stress (reviewed in Amores-Sanchez and Medina, 1999; Kelly and Wischmeyer, 2003; Lovat and Preiser, 2003). Furthermore, glutamine administration has also been shown to decrease muscle glutamine breakdown and enhance muscle protein synthesis (see Calder and Newsholme, 2002). Importantly, glutamine administration in humans has been shown to be safe with no harmful side effects (see Lacey and Wilmore, 1990; Garlick, 2001; Wilmore, 2001; Kelly and Wischmeyer, 2003).

Glutamine administration is beneficial following trauma in a number of ways. First, glutamine administration helps to maintain immune function (Newsholme, 2001; Watford, 2001; Mates et al., 2002). The carbon skeleton of glutamine can be used for glucose production and nucleic acid synthesis (Mates et al., 2002; Tapiero et al., 2002). These two factors are important for ensuring replication of immune cells. Secondly,

glutamine can be converted to glutamate by glutaminase. Glutamate dehydrogenase then removes an amino group and oxidizes glutamate to produce alpha-ketoglutarate which enters the Krebs cycle and acts to increase ATP synthesis. Finally, glutamine, via glutamate, is important for glutathione synthesis (Zubay et al., 1995).

1.4.2 Glutathione

Glutathione, L-γglutamyl-L-cysteinylglycine, is a tripeptide composed of glutamate, cysteine, and glycine (Figure 2). It is synthesized in two enzyme-catalyzed steps via γ-glutamylcysteine synthase (reaction 1), and glutathione synthase (reaction 2) (Meister and Anderson, 1983; Meister et al., 1986).

1) L-glutamate + L-cysteine + ATP ↔ L-γ-glutamyl-L-cysteine + ADP +Pi
2) L-γ-glutamyl-L-cysteine + ATP + glycine ↔ glutathione + ADP +Pi

Glutathione is the most abundant intracellular thiol (Lucas et al., 1998; Lucas et al., 2002), and serves to protect the cell from free radicals, reactive oxygen intermediates, and other toxic compounds (see Meister et al., 1986; Denno et al., 1996; Thorburne and Juurlink, 1996; Cooper and Kristal, 1997; Lucas et al., 1998; Amores-Sanchez and Medina, 1999; Prem et al., 1999; Lucas et al., 2002; Flaring et al., 2003; Johnson, 2003; Oehler and Roth, 2003). Glutathione acts as an electron donor to scavenge strong oxidants thereby reducing oxidative stress (reviewed in Juurlink, 1999). Glutathione (GSH) can reduce reactive oxygen species or oxidized proteins by donating electrons to produce oxidized glutathione (GSSG) and water via glutathione peroxidase. The oxidized glutathione can then be reduced by glutathione disulfide reductase using NADPH as an electron donor (see Meister and Anderson, 1983; Cooper and Kristal, 1997; Juurlink and Paterson, 1998; Amores-Sanchez and Medina, 1999; Lucas et al.,

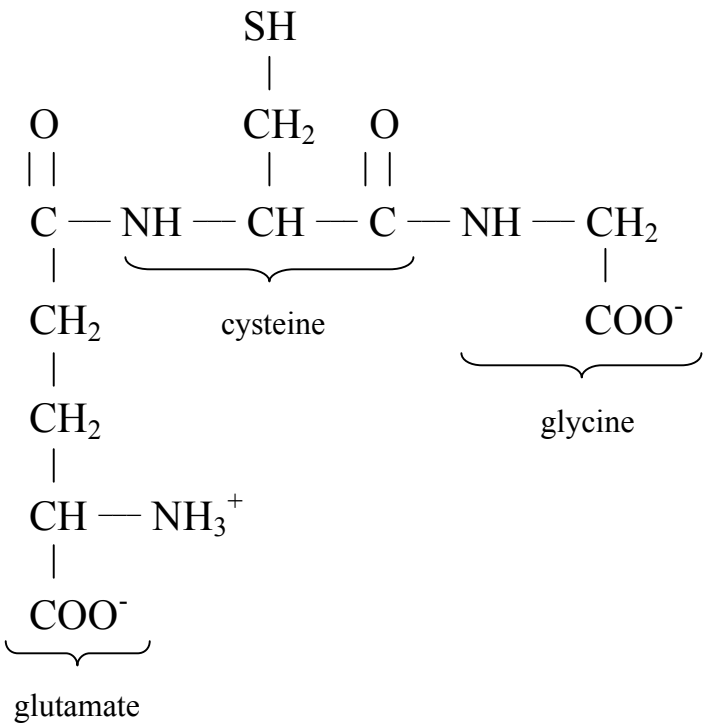


Figure 2. Stucture of glutathione (after The Merck Index, 1983).

2002). Additionally, glutathione can stop the chain of lipid peroxidation by reducing the α-tocopherol radical to α-tocopherol (see Cooper and Kristal, 1997; Juurlink, 1999).

Following spinal cord injury there is a significant decrease in glutathione within one hour (Kamencic et al., 2001; Lucas et al., 2002). Studies show that glutathione deficiency leads to oxidative stress and cell death in many tissues (see Meister, 1994; Cooper and Kristal, 1997). It has been shown that increasing intracellular glutathione allows cells to scavenge strong oxidants (reviewed in Cooper and Kristal, 1997; Juurlink, 1999); however, glutathione had poor penetration into cells (Meister et al., 1986; Cooper and Kristal, 1997). Meister (1986) suggested that "it would seem that an ideal way of increasing the glutathione levels would be to provide a derivative of glutathione that is effectively transported into the cell and that after transport is rapidly converted to glutathione within the cell". Others have shown that glutamine administration decreases glutathione depletion and leads to better recovery following trauma (Prem et al., 1999; Flaring et al., 2003). It has been shown that by administering glutamine after spinal cord injury, 75% of basal glutathione concentration is maintained compared to the 50% level found in spinal cord injured rats that did not receive glutamine (Rigley et al., 2002). It has also been demonstrated that by increasing glutathione production following spinal cord injury, there is a decrease in secondary injuries and an increased likelihood of regaining locomotory function in rats (Kamencic et al., 2001).

1.4.3 From glutamine to glutathione

Glutamine increases glutathione production in two main ways. Both ways involve glutamine's conversion to glutamate intracellularly. The first mechanism is that

increased intracellular glutamate enhances the ability of the glutamate/cystine antiporter to bring cystine into the cell (see Amores-Sanchez and Medina, 1999). Once inside the cell, one cystine molecule is converted to two molecules of cysteine (Juurlink, 1999). Therefore glutamine administration brings two of the three components of glutathione into the cell (Amores-Sanchez and Medina, 1999; Prem et al., 1999). The second mechanism involves the regulatory enzyme L-glutamyl-L-cysteine ligase. Glutathione inhibits L-glutamyl-L-cysteine ligase activity thereby limiting synthesis of glutathione. However, when intracellular glutamate levels are high the inhibition of L-glutamyl-L-cysteine ligase, by glutathione, is ameliorated allowing for increased synthesis of glutathione (see Juurlink, 1999).

1.5 Model system

Two muscles from the hind limb were used to study the effect of glutamine and SCI on rat skeletal muscle (see figure 3). The soleus and EDL muscles were chosen because both are locomotory muscles that are greatly affected by paraplegia. Furthermore, both muscles are supplied by nerves that originate from similar rootlets off the spinal cord. The soleus is innervated by the tibial nerve which arises from the sacral (S) level of the spinal cord at the level of S1 and S2. The EDL is innervated by the deep fibular nerve which arises from the lumbar (L) and sacral levels of the spinal cord at L5 and S1. Following SCI, both the soleus and the EDL experience a loss of innervation as well as decreased weight bearing (Kendall et al., 1993).

The soleus and EDL were also used because they are probably the most studied pair in the muscle biology literature (Gutman et al., 1972; Gupta et al., 1989; Termin et al., 1989; Esser et al., 1993; Hutchinson et al., 2001). Both act on the ankle joint and are

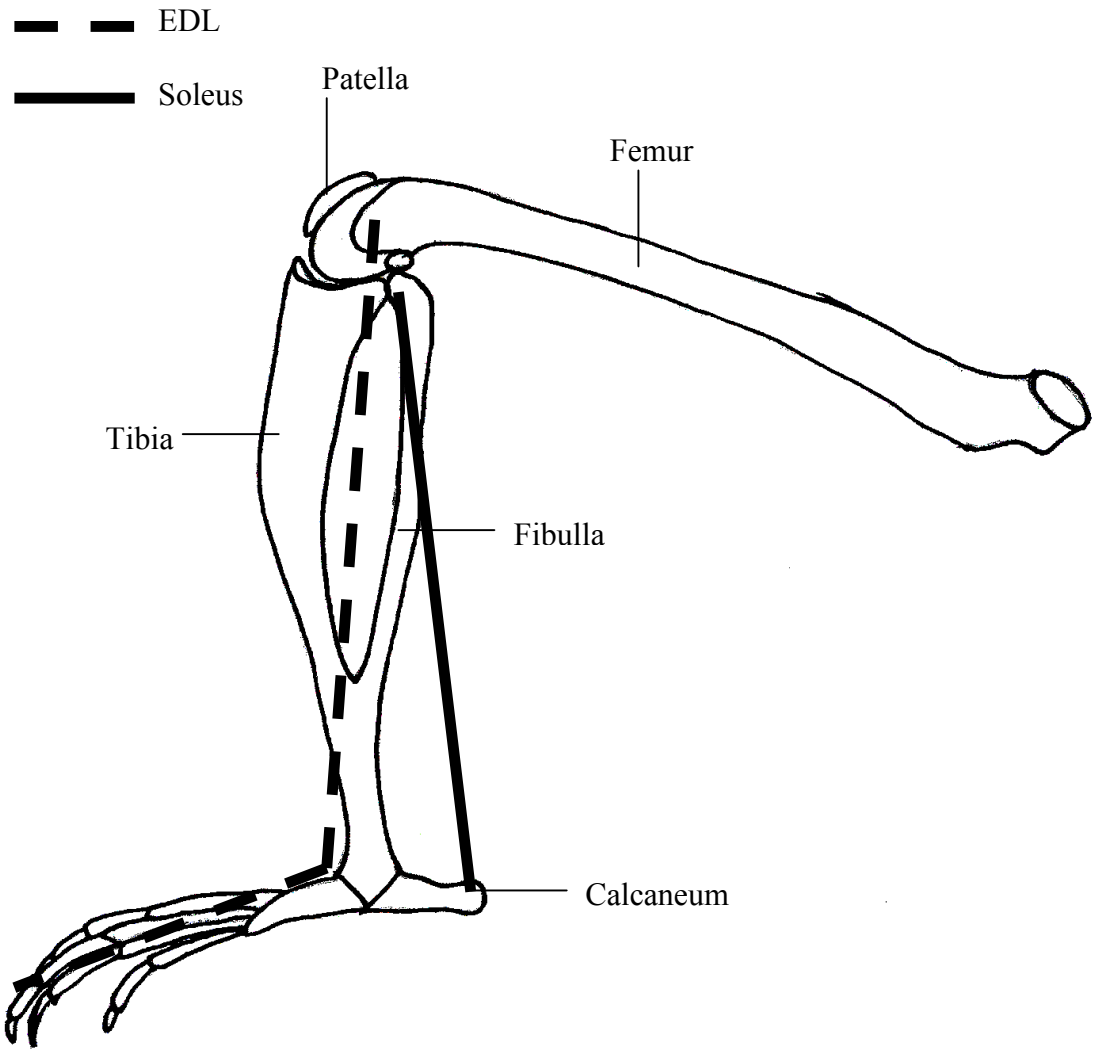


Figure 3. Location of EDL and soleus in the rat hindlimb.

similar in size. The soleus originates on the head of the fibula and the medial border of the tibia, and inserts into the calcaneus via the achilles tendon. Its action is to plantar flex the foot at the ankle. The EDL originates on the lateral condyle of the femur, passes under the extensor retinaculum, inserting onto the middle and distal phalanges of the toes. Its action is to extend the phalanges and dorsiflex the foot at the ankle (Hebel and Stromberg, 1976). The soleus muscle is a slow twitch extensor (Gutmann et al., 1972; Gupta et al., 1989; Jakubiec-Puka et al., 1990; Esser et al., 1993). It contains predominantly type 1 fibers with some expression of type 2a fibers (Carraro and Catani, 1983; Hamalainen and Pette, 1995; Staron et al., 1999; Talmadge et al., 1999) and is used for postural control or sustained tonic activity (Close, 1972; Pette and Vrbova, 1985; Baldwin and Haddad, 2002). In contrast, the EDL is a fast twitch flexor muscle (Gutmann et al., 1972; Gupta et al., 1989; Jakubiec-Puka et al., 1990; Esser et al., 1993; Roy et al., 1996). It contains almost entirely fast fibers including type 2x and 2b (Carraro and Catani, 1983; Staron et al., 1999) and is used for phasic movements (Close, 1972).

1.6 Introduction and hypothesis

Primary spinal cord injury (SCI) results from direct physical deformation of the spinal cord. Following the initial mechanical trauma, secondary spinal cord injury is caused by the resulting pathochemical and pathophysiological events including oxidative stress and inflammation (Anderson and Hall, 1993). Antioxidants play an important role in protecting cells from the damaging effects of oxidative stress, and may thereby help to reduce the amount of secondary damage following SCI. Glutamine may play an essential role in this process (Newsholme, 2001). Glutamine is the precursor of

glutathione which is an important antioxidant molecule that helps to prevent oxidative stress (Amores-Sanchez and Medina, 1999). It has been shown that by administering glutamine after spinal cord injury, 75% of basal glutathione concentration is maintained compared to the 50% level found in spinal cord injured rats that did not receive glutamine (Rigley et al., 2002). Also, increased glutathione production after spinal cord injury decreases secondary injuries and increased the likelihood of regaining locomotory function in rats (Kamencic et al., 2001).

Skeletal muscle phenotype is strongly influenced by neuromuscular activity, and studies have shown that change in neural input affects the myosin heavy chain (MyHC) composition of a muscle (Roy et al., 1991; Nakamura et al., 1997; Talmadge et al., 1999; Talmadge, 2000; Huey et al., 2001; Hutchinson et al., 2001). Mammalian skeletal muscles contain distinct muscle fiber types that are described as either slow or fast twitch depending on the type of myosin heavy chain (MyHC) isoforms they express. Neuronal disruption following spinal cord injury causes a shift in the expression of MyHC isoforms in rat, cat, and human fast and slow muscles (Roy et al., 1991; Talmadge et al., 1999; Talmadge, 2000; Huey et al., 2001).

The hypothesis tested is that **glutamine, as a precursor of glutathione, administration to spinal cord injured rats will lead to better functional recovery and a more preserved MyHC phenotype in locomotory muscles**.

1.7 Experimental Objectives

To test the experimental hypothesis the following objectives were addressed.

1. To determine if glutamine administration following SCI will help improve functional outcome in the rat.

2. To determine if glutamine treatment helps to preserve MyHC profile in the soleus and EDL muscles of the rat.
3. To determine if there is a correlation between improved functional outcome and a more preserved MyHC profile.

2. Methods

2.1 Animal Surgery

2.1.1 Animals

Thirty male Wistar rats (Charles River Laboratories, Wilmington, MA) were obtained 11 weeks of age at 238 to 351g for this study. Upon arrival animals were acclimatized for one week before surgery. Rats received standard rat chow and water *ad libitum* and were kept in a 12 hour light/dark cycle and 25ºC temperature controlled conditions. Following surgery, at weekly intervals, rats were weighed and behavioral testing was done. At six weeks post surgery the animals were killed by intracardiac perfusion (see section 2.3.1 for full details). All experiments were performed following the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 1510-130 Albert Street, Ottawa ON Canada K1P 5G4, http://www.ccac.ca).

2.1.2 Groups and Treatments

One group (n=8) contained healthy animals that did not undergo treatment of any kind. The second group (n=8) consisted of rats that received sham operations of laminectomies only. The third group (n=7) received SCI, but did not receive treatment. Finally, the experimental group (n=7) consisted of rats that received a SCI and glutamine injections. Glutamine was injected intraperitoneally at a dose of 1 mmol/kg which was determined to be the optimal dose at site of injury to increase glutathione concentrations in spinal cord tissue determined by high-pressure liquid chromatography (Rigley et al., 2002). The first dose of glutamine was administered one hour after

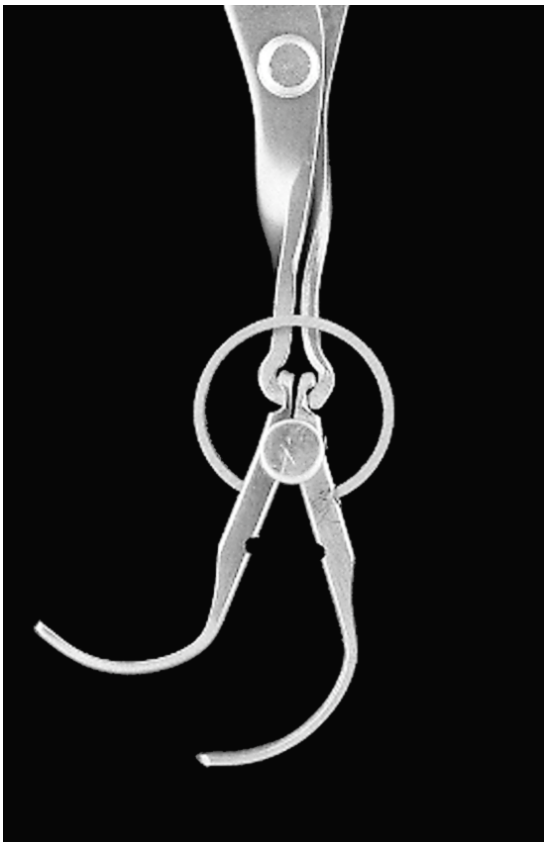
surgery. This was repeated every 12 hours to maintain blood glutathione levels as determined by a time response curve (Rigley et al., 2002) for one week post surgery (Benton et al., 2000).

2.1.3 Surgery

Each rat was placed in a chamber and anesthetized with five percent halothane (MTC Pharmaceuticals, Cambridge, ON) mixed with pure oxygen. Each animal was subsequently transferred to a table and maintained with two and a half percent halothane. The animal was shaved on its dorsal surface from the area at the base of its skull to the lumbar region and the area sterilized with Hibitane® (Chlorhexidine, Ayerst Laboratories, Montreal, ON) and 70% alcohol. A 0.05mg/kg dose of the analgesic Buprenorphrine® (Reckett Benckiser Pharmaceuticals Inc., Richmond, VA) was also injected subcutaneously.

Each animal was placed on its ventral surface and covered with a sterile cloth so that only the area to be operated upon was exposed. An incision was made from the fourth thoracic vertebra to the eighth thoracic vertebra in order to locate thoracic vertebrae five to seven. Superficial muscles from this area were cut and retracted, while deeper muscles attached to the vertebrae were removed. Then rongeurs were used to remove the spinal processes and laminae from these vertebrae (Figure 4B). Parts of the transverse processes were also removed so that a calibrated aneurism clip (modified Kerr-Lougheed clip, Walsh Manufacturing, Oakville, Ontario; Figure 4A) could be placed extradurally around the spinal cord at the level of the sixth thoracic (T6) vertebra (Figure 4C). The injury was performed at T6 in order to induce complete paraplegia without involving any injury to the forelimbs. The clip exerted a 30g force for five

A.



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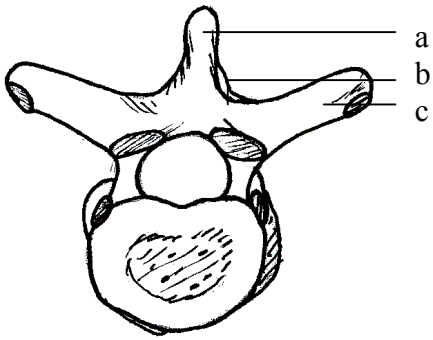

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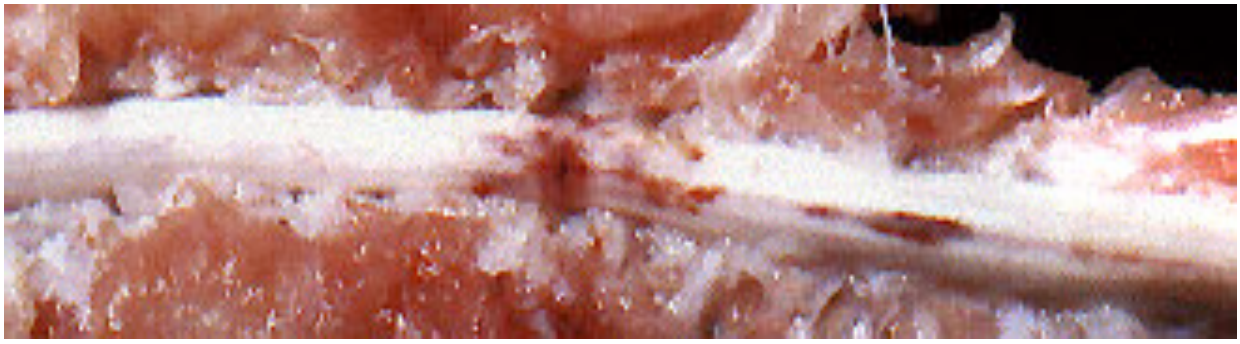

Figure 4. SCI model. **A)** Modified Kerr-Lougheed aneurism clip. **B)** Lumbar vertebrae. a= spinal process, b= lamina, c= transverse process **C)** Rat spinal cord 12 hours after clip surgery.

seconds before it was removed (Rivlin and Tator, 1978). Finally the muscle and fascia were closed with absorbable sutures, and staples were used to seal the skin.

Immediately following surgery, animals received a 3ml subcutaneous injection of Ringers solution (Presnell and Schreibman, 1997). Rats were then placed on a hot water bottle, filled with hot tap water, until the rats regained consciousness. Sham surgeries were performed in the same manner, except that the aneurism clip was not used. Tapered doses of Buprenorphrine® were injected at 12 hour intervals for three days.

2.1.4 Bladder Expression

Bladder expressions were performed on the animals that underwent the spinal cord injury as they lost the ability to urinate. Bladder expressions were performed manually by squeezing the rat's bladders prompting them to urinate. This was done three times a day until function returned, usually around two weeks, or the animal was killed.

2.2 Behavioral Testing

Rats were weighed and tested weekly following surgery.

2.2.1 Angle Board Method

Rats were assessed using the angle board method of Rivlin and Tator, 1977. This is a behavioral task that assesses motor function by determining the maximum angle that a rat can maintain its position without falling off of an angled board (Figure 5). Rats started off at an angle of 25º to the horizontal. The board was then increased at five

Figure 5. Angle board method used to determine the maximum angle that a rat can maintain his position on an angled board without falling off. Photo courtesy of Elizabeth Schültke.

degree increments until the rat could no longer maintain his position or a maximum angle of 55º was reached.

2.2.2 BBB Score

The Basso-Beattie-Bresnahan (BBB) open field locomotor rating scale (Basso et al., 1995) rates a rat's ability to walk using a twenty-one point scale that measures locomotor recovery. Each animal receives a score that reflects its ability to perform certain movements including movement of the three hind limb joints, sweeping motions with the hind limb, weight support, paw placement, fore limb- hind limb coordination, toe clearance, and tail position. Scores given by two independent observers were almost always identical. When there was a discrepancy in scoring, by one point, the given score was reached by consensus.

On the BBB scale zero equals total paralysis or no hind limb movement, and a score of twenty-one signifies normal locomotion (Figure 6). As BBB scores increase, rats display an increased range of movement at the hip, knee, and ankle joints until they are able to take occasional steps, a score of ten. At that point, higher scores indicated increased frequency of stepping, weight support, proper position of the foot, hindlimb-forelimb coordination, toe clearance, ability to keep the tail raised, and trunk stability. For example, a score of one indicates that there is slight movement at one or two joints, and a score of two indicates that a rat has extensive movement of one joint and possibly slight movement of another joint. A score of three indicates extensive movement at two joints (Basso et al., 1995). A rat scoring four on the BBB scale has slight movement of all three hindlimb joints. Scores of five and six indicate increased movement of the joints, while a score of seven signifies that a rat has extensive movement at all three

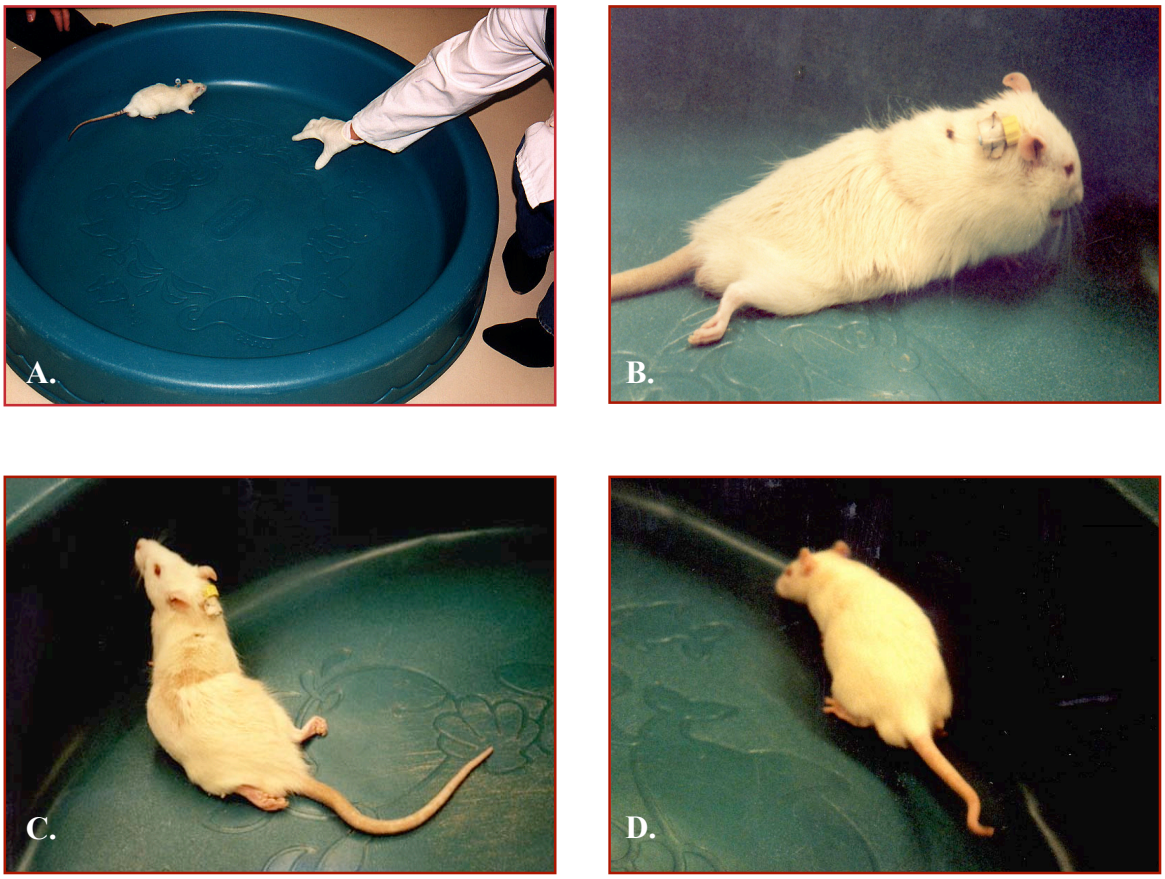

Figure 6. The Basso-Beattie-Bresnahan locomotor rating scale. A) Testing is performed in a plastic pool for four minutes, and each animal is scored. B) This rat would receive a score of zero indicating that there is no observable hindlimb movement. C) A score of eight indicates that a rat displays either a sweeping movement with no weight support, or plantar placement of a paw without weight support. D) A rat showing occasional plantar steps with weight support, but no forelimb-hindlimb coordination received a score of ten. Photo courtesy of Elizabeth Schültke.

hindlimb joints. A score of eight indicates sweeping or plantar placement of the paw without weight support. When a score of nine is achieved, the rat has either plantar placement of the paw with weight support while standing, or weight supported dorsal stepping but no stepping with the paw placed on its plantar surface. A score of ten indicates that a rat can take occasional weight supported steps on the plantar surface of his paw, but that he is not displaying any forelimb-hindlimb coordination. When a score of 12 is achieved rats begin to display forelimb-hindlimb coordination. A score of 16 implies that a rat is displaying consistent plantar stepping and forelimb-hindlimb coordination, and frequent toe clearance although his paw position is not parallel during the stepping process, he is unable to hold his tail up, and his trunk is unstable. Finally, a score of 21 would indicate normal locomotion. For a more detailed description of the BBB locomotor rating scale, see Basso et al., 1995.

2.3 Tissue Collection

2.3.1 Perfusion

Rats were anesthetized with five percent halothane. An incision was made in the skin just below the xiphoid process. This was extended through the abdominal muscle and fascia along the ribs on either side to the midaxillary line. Then, the diaphragm was cut away from the ventral thoracic wall allowing the incision to be extended along the midaxillary line rostrally so that the entire ventral thoracic wall could be reflected upward exposing the heart. A blunt end intravenous needle was used to puncture the left ventricle allowing it to enter the aorta where it was held in place with hemostats. After

opening the right atrium to allow the draining of blood, a cold, non-heparinized, saline drip was attached to the intravenous needle and allowed to start dripping. When all of the blood had been drained from the rat, the intravenous drip was stopped and removed. At this time both the right and left hind legs were removed from the animal. Subsequently, the rat was perfused with four percent paraformaldehyde for other studies.

2.3.2 Collection of Skeletal Muscle

EDL and soleus muscles were removed from each limb. Each muscle was then weighed, and frozen in isopentane cooled in liquid nitrogen (Brumback and Leech, 1984). The diaphragm from several control animals was also frozen in isopentane cooled in liquid nitrogen for biochemical uses as a control muscle in SDS-PAGE gels. Tissue samples were stored at -80ºC.

2.4 Separation of skeletal muscle MyHC isoforms

Rat skeletal muscle MyHC isoforms were separated using a protocol modified from the methods of Talmadge and Roy, 1993. The modifications are described in the following.

2.4.1 Myofibril purification

All homogenates, suspensions, and buffers were kept on ice during the following procedure. Tissue was ground using a mortar and pestle in dry ice. Tissue was then separated into two or more centrifuge tubes and 0.8ml of Buffer #1 (250 mM sucrose, 100mM KCL, 5 mM EDTA, 10mM Tris-base, pH 6.8) was added to each tube. The tissue was homogenized using a pestle in 1.5ml G-tube™ (Bio Plas, Inc, California) by

hand for an additional minute. The sample was then spun at 6000RPM for 10 minutes at four ºC, as were all other centrifugations done at four ºC, and the supernatant discarded. The sample was re-suspended in Buffer #2 (150 mM KCL, 10 mM Tris, 0.5% Triton-X 100, pH 6.8), homogenized for a minute, as in the preceding sentence, and then centrifuged at 3000RPM for three minutes. The sample was then re-suspended in Buffer #2, homogenized for a minute, and then centrifuged at 3000RPM for three minutes a second time. The sample was then washed in 0.8ml Buffer #3 (150 mM KCL, 10 mM Tris, pH 7.0), homogenized for a minute, vortexed for 20 seconds, then centrifuged at 3000RPM for three minutes. The sample was then washed in Buffer #3, homogenized for a minute, and centrifuged at 3000RPM for three minutes three additional times discarding supernatant each time after the sample was centrifuged. Enough Buffer #3 was then added until the pellet of purified myofibrils was just submerged. The sample was homogenized by hand for one minute in a centrifuge tube, centrifuged for 30 seconds, and then stored at -80ºC until electrophoresed.

2.4.2 Preparation of sample to load into gel

Muscle samples were diluted 1:1 with glycerol and then mixed with an equal volume of protein stock buffer (28% glycerol, 2.8% SDS, 1 M beta-mercaptoethanol, 4X stacking buffer, bromophenol blue). The 4X stacking buffer had a pH value of 6.8, and contained TRIS base, 10%SDS, and deionized water. Samples were contained in 1.5ml centrifuge tubes and placed in boiling water for five minutes. Samples were then centrifuged for 45 seconds and the supernatant drawn off and the pellet discarded.

2.4.3 Gels for MyHC separation

Eight percent acrylamide mini separating gels, 30% glycerol content, with five percent acrylamide stacking gels were used. For the eight percent portion of the gel, glycerol stock (68.3%) was mixed with premixed 40% acrylamide (Bio-Rad, Ontario, catalogue #161-0140) and 2% bis (Bio-Rad, Ontario, catalogue #161-0142). After 4X separating buffer (TRIS base, 10% sodium dodecyl sulfate (SDS), deionized water, pH=8.8) and 1 M glycine stock was added, the mixture was degassed in a flask for 15 minutes. The mixture was then cooled on ice and TEMED, 1,2-DI-(dimethylamino)ethane, and 10% ammonium persulfate (APS) were added. The gel was then poured, overlaid with distilled water, and set aside to polymerize for 30-60 minutes. For the stacking gels 50% glycerol sock was mixed with premixed 40% acrylamide (Bio-Rad, Ontario, catalogue #161-0140) and 2% bis (Bio-Rad, Ontario, catalogue #161-0142) stock, 4X stacking buffer (as mentioned previously), 100mM EDTA, and 10% SDS. The mixture was degassed for 15 minutes. During this time, the water overlay was removed from the separating gels which were then blotted dry with filter paper. TEMED and 10% APS were added to the stacking gel which was poured on the separating gel and 10 well combs (0.75mm) inserted into the stacking gel.

2.4.4 Gel Electrophoresis

The Mini-PROTEAN three cell gel electrophoresis apparatus (Bio-Rad, Ontario) was set up and predetermined amounts, to produce optimum visualization, of prepared myofibril samples were loaded into gel lanes with a Hamilton syringe (Hamilton Company, Reno, Nevada). A broad range SDS-PAGE molecular weight standard (Bio-

Rad, Ontario, catalogue # 161-0317) with a marker for myosin at 200 000 daltons was also loaded into the gel. The lower chamber of the apparatus was filled with lower running buffer (50 mM Tris base, 75 mM glycine, 0.05% SDS), and the upper chamber was filled with upper running buffer (0.1 M Tris base, 150 mM glycine, 0.1% SDS and 0.1% beta-mercaptoethanol). The entire apparatus was then placed into a walk-in refrigerator, four degrees Celsius, and attached to a power supply. The gel was run at 100 V for approximately 30 hours. When complete, the gel was removed from the apparatus and rinsed with distilled water. The gel was then stained with 0.25% Coomassie blue stain for 15 minutes, and then de-stained using 10% methanol and 10% acetic acid, until the MyHC bands were visible and the background staining was minimized. Gels were then stored in 10% glycerol and subsequently wrapped in cellophane membrane (Bio-Rad, Ontario) and allowed to air dry for two days before being filed.

2.5 Digital photography

Gels were placed on a sheet of clear plastic on top of a white light source under a digital camera connected to a computer. Scion Image (Frederick, Maryland, USA) was used to visualize pictures of the gels.

2.6 Measuring density of MyHC isoforms

Densities of MyHC isoforms were measured using Scion Image as employed by other researchers (Reidler, 2000; Kuscu et al., 2003). First background staining was removed from the entire gel. Next individual lanes were selected for density analysis. The density of each lane was then plotted as a series of peaks, and the area under each

peak was measured. Finally, calculations were done to determine the relative percentage of each MyHC isoforms present in each lane.

2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 3.0 statistical software (GraphPad Software, San Diego, CA). Analyses of variance (ANOVAs) were performed followed by Bonferroni post-hoc tests to determine where significant differences existed when there was a significant interaction between main effects. Arcsine transformations were performed where the data were expressed as percentages (Zar, 1999). Linear regressions were performed to determine the correlation between myosin isoforms and BBB scores, as well as between myosin isoforms and angle board scores. Parametric statistical tests were used to determine the significance of the BBB Locomotor scores as suggested by Scheff et al. (2002), and as employed by other researchers (Basso et al., 1995; Schultke et al., 2003). A p-value of $p<0.05$ was considered to be statistically significant. Values are expressed as mean and standard deviation (SD).

3. Results

3.1 Effect of SCI and treatment on rat weight

3.1.1 Change in body weight

Healthy and sham rats were not significantly (two-way ANOVA, $p>0.05$, Figure 7, Table 1) different from each other at any time point. Untreated rats were significantly different from sham rats at weeks one and two ($p<0.05$) and weeks three through to six ($p<0.001$). Untreated rats were also significantly different than healthy rats at week two ($p<0.05$) and weeks three, four, five, and six ($p<0.001$); however, untreated rats were not significantly different ($p>0.05$) from healthy rats one week after surgery. Treated rats were not significantly different ($p>0.05$) from healthy or sham rats one week after surgery, but did have a significantly ($p<0.05$) lower percent change in body weight two weeks after surgery. Five weeks after surgery, the percent change in weight was not significantly different ($p>0.05$) between treated and sham rats. Nonetheless, treated rats were significantly different from sham rats at weeks three ($p<0.05$), four ($p<0.01$), and six ($p<0.05$). Treated rats were significantly different from healthy rats at weeks three through to six ($p<0.01$). Treated and untreated rats were not significantly different from one another in the first four weeks after injury, but had significantly different percent changes in weight at week five ($p<0.01$), and week six ($p<0.001$).

3.1.2 Change in skeletal muscle weight

Soleus and EDL muscles were weighed six weeks post surgery after the animals were killed and the muscles excised.

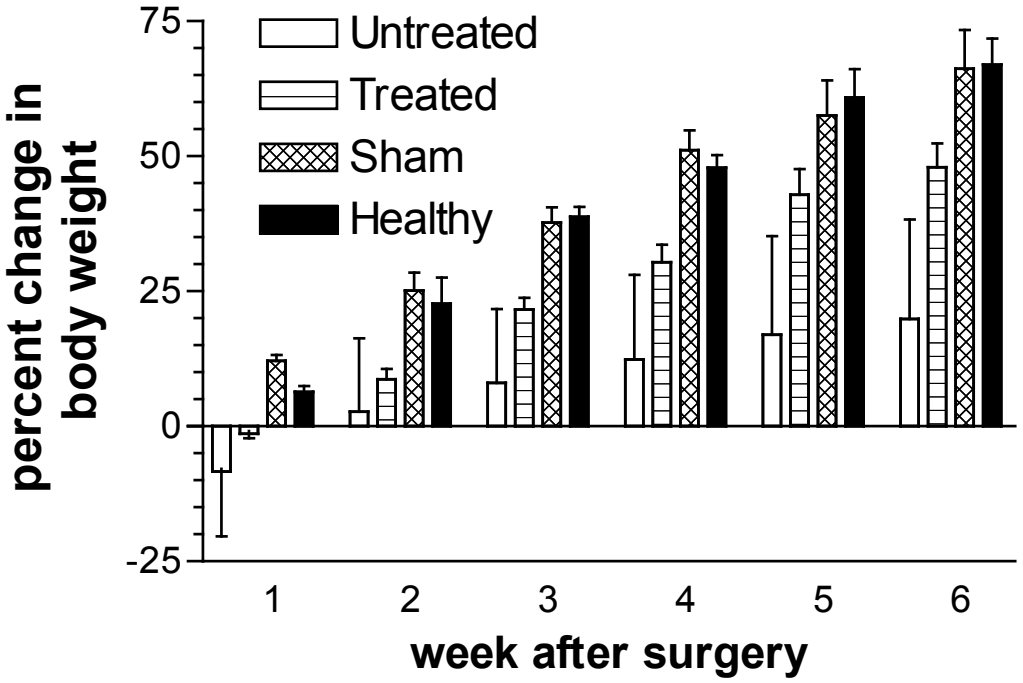

Figure 7. Effect of glutamine treatment on the change in percent body weight relative to baseline weight at the time of surgery. During the first week following surgery both treated and untreated rats experienced weight loss. Within the next five weeks, all groups of rats gained weight although treated and untreated rats weighed less than healthy and sham rats. For treated and untreated groups n= 7, for healthy and sham groups n= 8. Each bar equals mean and SD.

Group	Week	Treated	Sham	Healthy
Untreated	1	NS	p<0.05	NS
	2	NS	p<0.05	p<0.05
	3	NS	p<0.001	p<0.001
	4	NS	p<0.001	p<0.001
	5	p<0.01	p<0.001	p<0.001
	6	p<0.001	p<0.001	p<0.001
Treated	1		NS	NS
	2		p<0.05	p<0.05
	3		p<0.05	p<0.01
	4		p<0.01	p<0.01
	5		NS	p<0.01
	6		p<0.05	p<0.01
Sham	1			NS
	2			NS
	3			NS
	4			NS
	5			NS
	6			NS

Table 1. Significant differences in percent change in body weight of treated, untreated, healthy, and sham rats week one through to six.

3.1.2.1 Soleus

At six weeks post surgery the soleus muscles from untreated rats weighed significantly less (one-way ANOVA, $X=0.0575 \pm 0.0069$, $p<0.001$) than all other groups (Figure 8). In rats treated with glutamine, the soleus muscles made up a significantly ($X=0.0752 \pm 0.0063$, $p<0.001$) greater percentage of body weight compared to untreated rats. However, the soleus muscles from treated rats made up a significantly lower percentage of body weight compared to healthy ($X=0.0935 \pm 0.0084$, $p<0.001$) and sham ($X=0.0883 \pm 0.0086$, $p<0.05$) rats. Healthy and sham animals were not significantly ($p>0.05$) different from one another.

3.1.2.2 EDL

At six weeks post surgery, the EDL percentage of body weight was not significantly (one-way ANOVA, $p>0.05$, Figure 9) different between any of the four groups.

3.2 Behavioral analysis of hind limb strength and locomotor recovery

Behavioral analysis was performed weekly post surgery to determine hindlimb strength and locomotor recovery outcome.

3.2.1 Angle board method

One-way ANOVAs with Bonferroni post-tests were performed for each week. Healthy and sham groups of rats were able to maintain an angle of 55º, the upper limit of the measurement, throughout the six weeks and were not significantly ($p>0.05$) different from one another at any time point. Untreated rats were unable to maintain a minimum

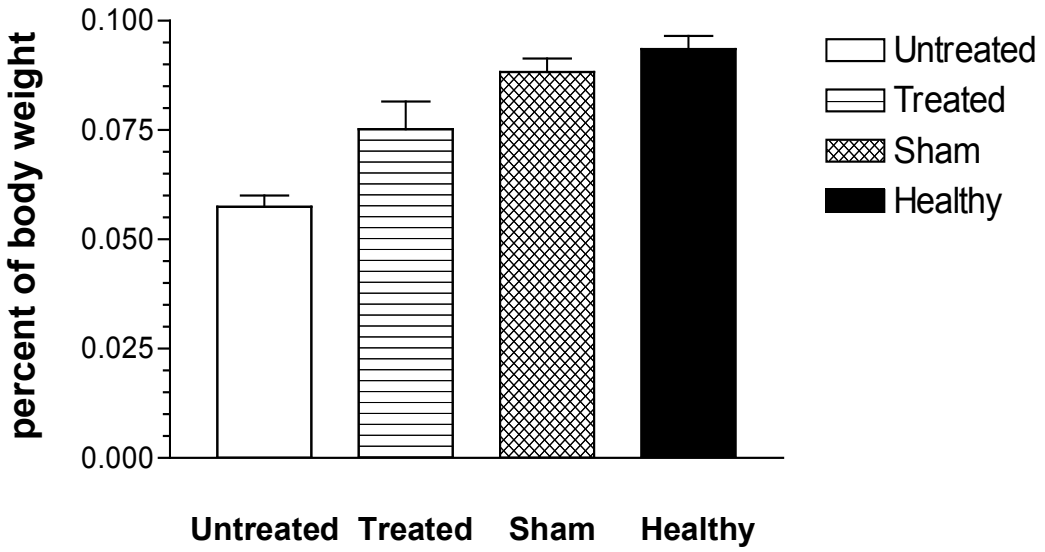

Figure 8. Soleus muscle as percentage of body weight. Soleus muscles from week six of glutamine treated animals maintain a greater percentage of body weight than untreated animals. For treated and untreated groups n=7, for healthy and sham groups n=8. Each bar equals mean and SD.

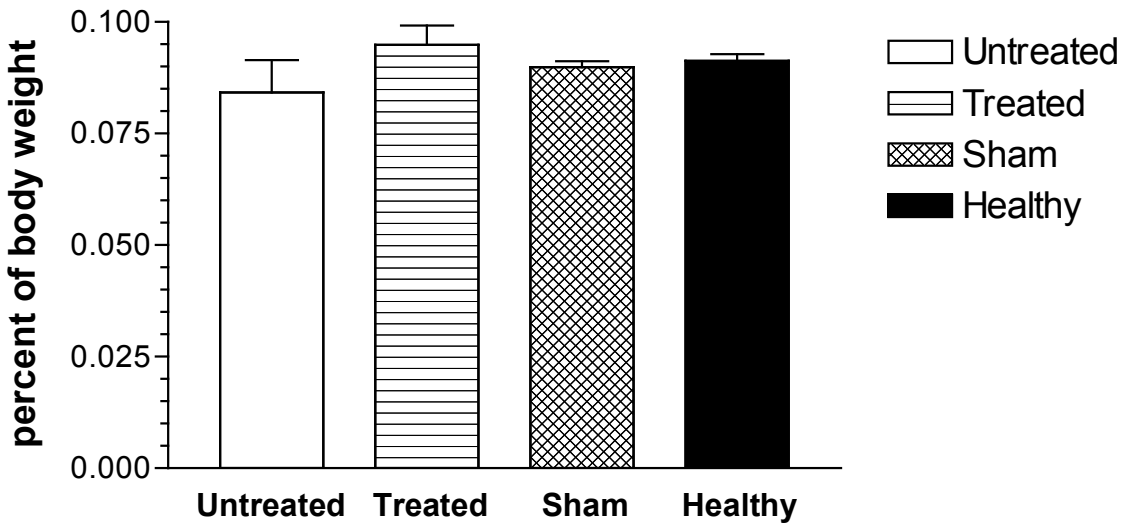

Figure 9. EDL muscle as percentage of body weight. EDL percentage of body weight is similar for all groups at six weeks post surgery. For treated and untreated groups n=7, for healthy and sham groups n=8. Each bar equals mean and SD.

angle of 25º for the first two weeks post surgery. All rats unable to maintain a minimum angle of 25º were given a score of 20. In the following weeks untreated rats were able to maintain mean angles ± SD of 11.43 ± 14.35, 12.86 ± 16.29, 12.86 ± 16.29, 20.71 ± 14.56 at weeks three, four, five, and six respectively (Figure 10). Treated rats started showing signs of returning hind limb strength as soon as the first week post surgery. For weeks one through six the means and SDs of this group were as follows 5.00 ± 13.23, 10.00 ± 18.03, 32.14 ± 9.06, 36.43 ± 8.52, 35.71 ± 8.53, 36.43 ± 7.48 (Figure 10). Untreated and treated rats were not significantly different at weeks one, two, but were significantly different at weeks three, four, five ($p<0.001$), and six ($p<0.01$). Untreated and treated groups were significantly ($p<0.001$) different from healthy and sham groups at all time points.

3.2.2 BBB score

One-way ANOVAs with Bonferroni post-tests were performed for each week. Healthy and sham groups of rats received consistent scores of 21 throughout the study indicating that they had normal locomotion. Treated and untreated groups had BBB scores significantly (one-way ANOVA, $p<0.001$) lower than healthy and sham groups at all time points. Untreated rats mean BBB scores were significantly lower than those of treated rats at one week ($p<0.01$) and weeks two through to six ($p<0.001$) post surgery. Untreated rats scores ± SD during weeks one to six respectively are as follows 0.43 ± 0.79, 2.43 ± 2.44, 1.71 ± 2.81, 2.86 ± 2.91, 3.43 ± 3.05, and 2.86 ± 2.27. Treated rats received scores ± SD of 4.29 ± 3.73, 9.43 ± 3.05, 8.57 ± 2.82, 9.29 ± 3.20, 9.57 ± 3.31, and 9.57 ± 3.31 throughout weeks one to six respectively (Figure 11).

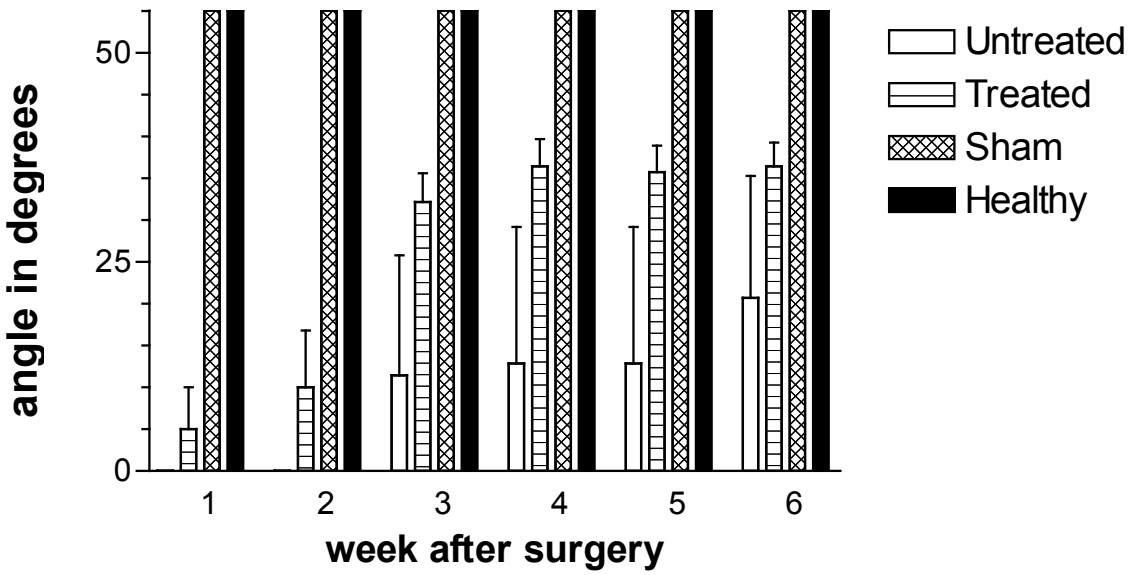

Figure 10. Weekly angle board scores of rats for six weeks. Glutamine treated rats were able to maintain greater angles on an angle board than untreated rats. Untreated rats were not able to maintain their positions on an angle board the first two weeks post surgery. For treated and untreated groups n=7, for healthy and sham groups n=8. Each bar equals mean and SD.

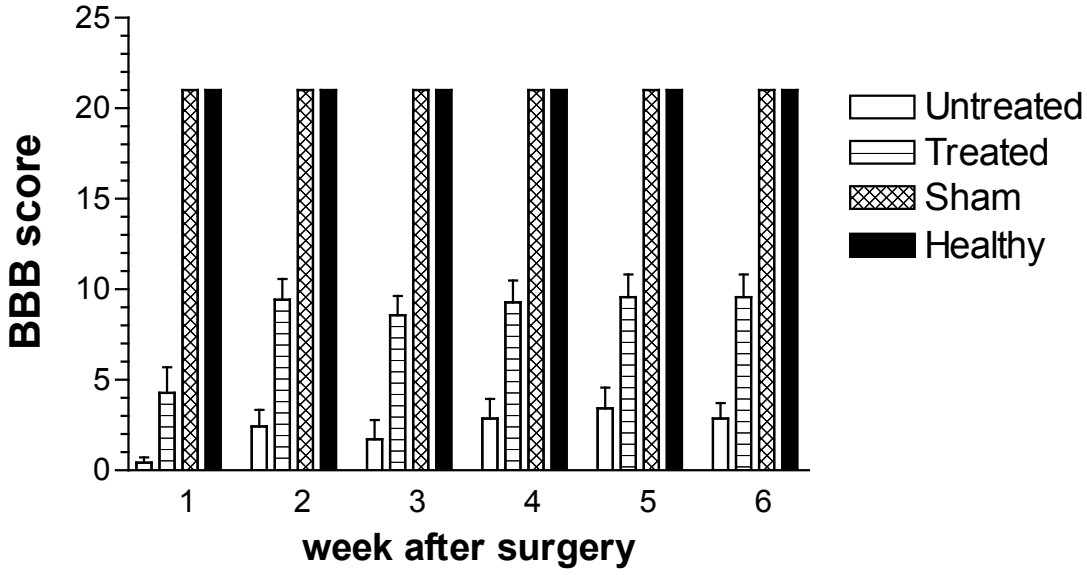

Figure 11. Weekly BBB scores of rats for six weeks. Glutamine treated rats received higher BBB scores than untreated rats at all time points. For treated and untreated groups n=7, for healthy and sham groups n=8. Each bar equals mean and SD.

3.3 Effect of SCI and treatment on rat MyHC isoforms

3.3.1 Soleus

Table 2A displays the mean and SDs of MyHC isoforms as a percent of the total myosin for the four groups. A two-way ANOVA comparing MyHC isoforms and percentage of total myosin for the four different groups found that a significant ($p<0.0001$) interaction took place between the treatment groups and MyHC isoforms expressed in the soleus muscle. Bonferroni post-tests were performed to determine where the significant differences occurred (Table 2B). Glutamine treated rats had a more preserved MyHC phenotype than untreated rats. See Figures 12A, 12B and 12C for pictures of SDS-PAGE of the MyHC isoforms present in the soleus muscle of the various groups. Note that there are different sequences of treatment groups in each set of pictures. The data was presented in this way because each set of pictures are from the same gel and the samples were run in the order depicted. See section 4.3.3 for a discussion regarding differences in MyHC isoform distribution in diaphragm samples.

3.3.2 EDL

Table 3A displays the mean and SDs for the various groups. A two-way ANOVA comparing MyHC isoforms and percentage of total myosin for the four different groups found that a significant ($p<0.0001$) interaction took place between the treatment groups and MyHC isoforms expressed in the EDL muscle. Bonferroni post-tests were performed to determine where the significant differences occurred (Table 3B). Healthy rats were not significantly ($p>0.05$) different compared to rats from the sham group. Untreated rats did not express any significant ($p>0.05$) differences in the

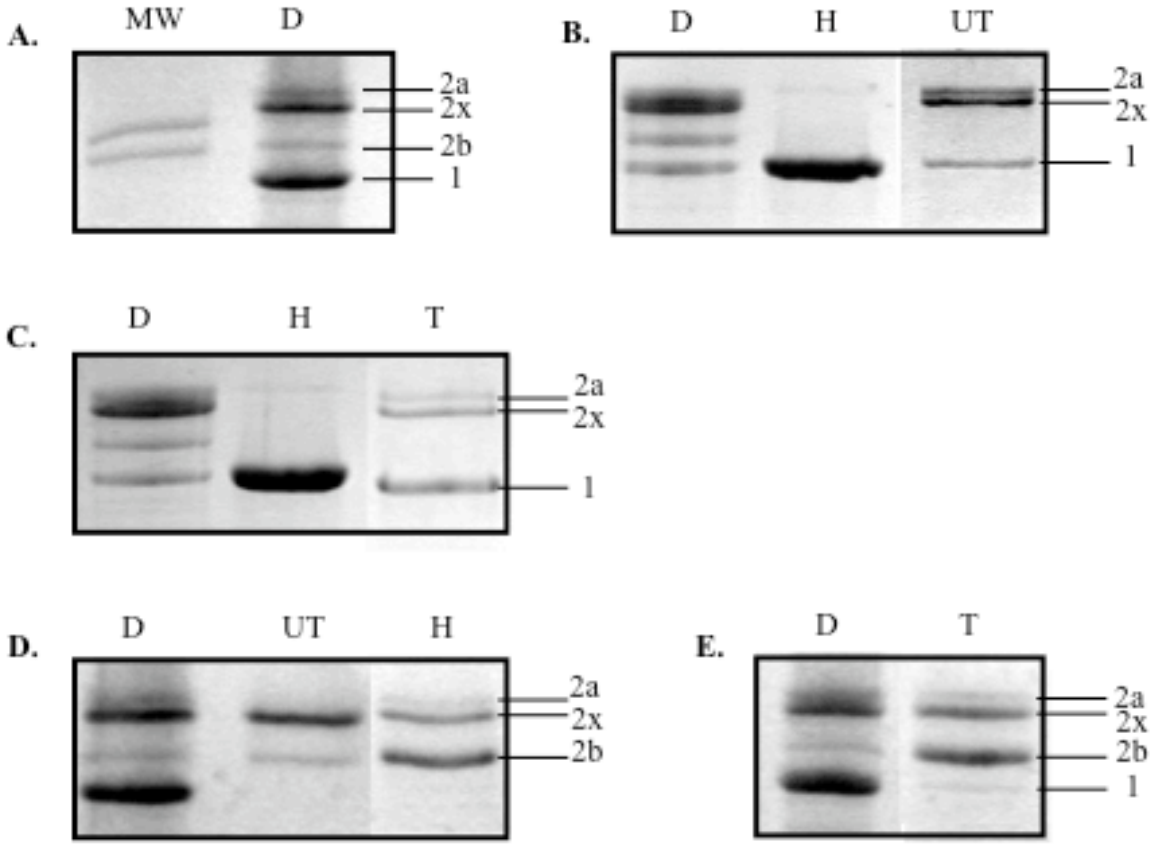

Figure 12. Pictures of SDS-PAGE gels showing rat MyHCs from soleus and EDL muscles of various treatment groups. Each group of pictures was run in the same gel. Molecular weight markers and diaphragm samples were run in every gel. The molecular weight marker contained two different isoforms of myosin from rabbit skeletal muscle. Note that myosin samples from the diaphragm contain all four MyHCs. The slowest migrating band, highest band on the gel, represents MyHC 2a. Below it is MyHC 2x, followed by MyHC 2b, and the fastest migrating band, lowest band on gel, is MyHC 1. **A)** Picture showing that rat diaphragm lines up with the molecular weight marker at 200,000 daltons. **B)** Myosin from the soleus muscle. Myosin from healthy animals displays predominantly type 1 MyHC, with some type 2a MyHC. Conversely, myosin from untreated rats shows much less type 1 MyHC, and also expresses higher levels of 2a and 2x MyHCs. **C)** Myosin from the soleus muscle. Myosin from rats treated with glutamine displays a MyHC profile similar to that of untreated rats, but displays more type 1 MyHC compared to type 2a or 2x MyHCs. **D)** Myosin from the EDL muscle. Healthy rats display the greatest expression of type 2b MyHC, with some type 2x, and less 2a MyHC. Untreated rats show a decreased expression of 2b MyHc and an increased expression of 2x MyHC. **E)** Myosin from the EDL muscle. Rats treated with glutamine have an increased expression of 2b MyHC and a decreased expression of 2x MyHC compared to untreated rats. MW= molecular weight marker, D= diaphragm, H= healthy rat, UT= untreated rat, T= treated rat.

A.

Soleus		Percent of total myosin			
Group	**n**	**2a**	**2x**	**2b**	**1**
Untreated	7	33.90 ± 6.90	38.33 ± 9.31	0.09 ± 0.23	27.69 ± 5.69
Treated	7	15.01 ± 7.49	16.54 ± 16.71	0.57 ± 1.47	67.87 ± 23.72
Sham	8	1.06 ± 2.10	0.56 ± 1.59	0.00 ± 0.00	98.38 ± 3.05
Healthy	8	5.11 ± 4.86	0.90 ± 1.60	0.00 ± 0.00	93.99 ± 5.66

B.

Group	MyHC	Treated	Sham	Healthy
Untreated	**1**	p<0.001	p<0.001	p<0.001
	2a	p<0.05	p<0.001	p<0.001
	2x	p<0.01	p<0.001	p<0.001
	2b	NS	NS	NS
Treated	**1**		p<0.001	p<0.01
	2a		p<0.05	NS
	2x		p<0.05	p<0.01
	2b		NS	NS
Sham	**1**			p<0.05
	2a			p<0.05
	2x			NS
	2b			NS

Table 2. Soleus MyHC isoform percentage of total myosin at six weeks post surgery. **A)** Values shown are mean and SD. **B)** Significant differences in MyHC isoforms in the soleus of treated, untreated, sham, and healthy groups. There is a significant interaction (two-way ANOVA, $p<0.0001$) between treatment group and MyHC isoform expression; therefore, Bonferroni posttests were performed. Glutamine treated rats display a more preserved myosin phenotype in the soleus muscle than untreated rats.

A.

EDL		Percent of total myosin			
Group	**n**	**2a**	**2x**	**2b**	**1**
Untreated	7	0.36 ± 0.96	73.50 ± 7.37	25.97 ± 7.34	0.17 ± 0.53
Treated	7	2.21 ± 4.01	65.51 ± 20.52	31.96 ± 17.72	0.33 ± 0.87
Sham	8	13.57 ± 8.13	31.69 ± 5.51	51.22 ± 9.14	3.52 ± 4.62
Healthy	8	11.20 ± 3.11	31.46 ± 7.72	54.99 ± 9.40	2.36 ± 2.71

B.

Group	MyHC	Treated	Sham	Healthy
Untreated	**1**	NS	NS	NS
	2a	NS	p<0.001	p<0.001
	2x	NS	p<0.001	p<0.001
	2b	NS	p<0.001	p<0.001
Treated	**1**		NS	NS
	2a		p<0.01	p<0.01
	2x		p<0.001	p<0.001
	2b		p<0.05	p<0.01
Sham	**1**			NS
	2a			NS
	2x			NS
	2b			NS

Table 3. EDL MyHC isoform percentage of total myosin at six weeks post surgery. **A)** Values shown are mean and SD. **B)** Significant differences in MyHC isoforms in the EDL of treated, untreated, sham, and healthy groups. There is a significant (two-way ANOVA, $p<0.0001$) interaction between treatment group and MyHC expression. Bonferroni posttests were performed to determine where significant differences exist. Treated and untreated rats are not different from each other, nor are healthy and sham rats. However, both treated and untreated rats show significant differences in MyHC expression compared to healthy and sham rats.

percentages of MyHC isoforms expressed compared to treated rats. However, there were significant differences in MyHC percentage of total myosin between treated/untreated, and healthy/sham groups in the expression of type 2 MyHCs. More specifically, treated and untreated rats have less 2a and 2b MyHCs and more type 2x MyHC than healthy and sham rats. No significant difference existed between type 1 MyHCs. See Figures 12D and 12E for pictures of SDS-PAGE of the MyHC isoforms present in the EDL muscles of the various groups.

3.4 Correlation between behavioral scores and MyHC isoform expression

Linear regressions were performed to determine if improved behavioral scores were correlated to more preserved MyHC expression. For example, in the soleus muscle which contains a majority of type 1 MyHCs, linear regressions were performed to see if better behavioral scores were related to a greater percentage of type 1 MyHC in the soleus muscle. Alternatively, in the EDL which contains mainly type 2b MyHCs a linear regression analysis was performed to see if better behavioral scores were accompanied by greater amounts of type 2b MyHC expression.

Linear regressions were performed comparing the percentage of type 1 MyHC present in rat soleus muscle to the six week angle board scores, and the six week BBB scores attained by all rats (n=30). Similarly, linear regressions were performed comparing the percentage of type 2b MyHCs present in rat EDL muscles to the six week angle board, and six week BBB scores attained by these same rats to see if there were any correlations between these parameters. The data from these results show that better locomotion and greater hindlimb strength are also related to a more preserved MyHC phenotype.

3.4.1 Angle board method

There was a significant (linear regression, r^2= 0.8686, p<0.0001, Figure 13) correlation between the percent of type 1 MyHC isoform present in the soleus muscle and angle board score received by all rats involved in the study. Similarly, a significant (Linear Regression, r^2= 0.6084, p<0.0001, Figure 14) correlation was found between the percent of type 2b MyHC isoform present in the EDL muscle and the angle board score received by all rats in the study.

3.4.2 BBB score

When comparing the percent of type 1 MyHC isoform present in the soleus muscle and the BBB scores received by rats via linear regression a significant (r^2= 0.8708, p<0.0001, Figure 15) correlation was found. Likewise a significant (r^2= 0.6290, p<0.0001, Figure 16) correlation was found when comparing the percent of type 2b MyHC isoform expressed and BBB scores achieved.

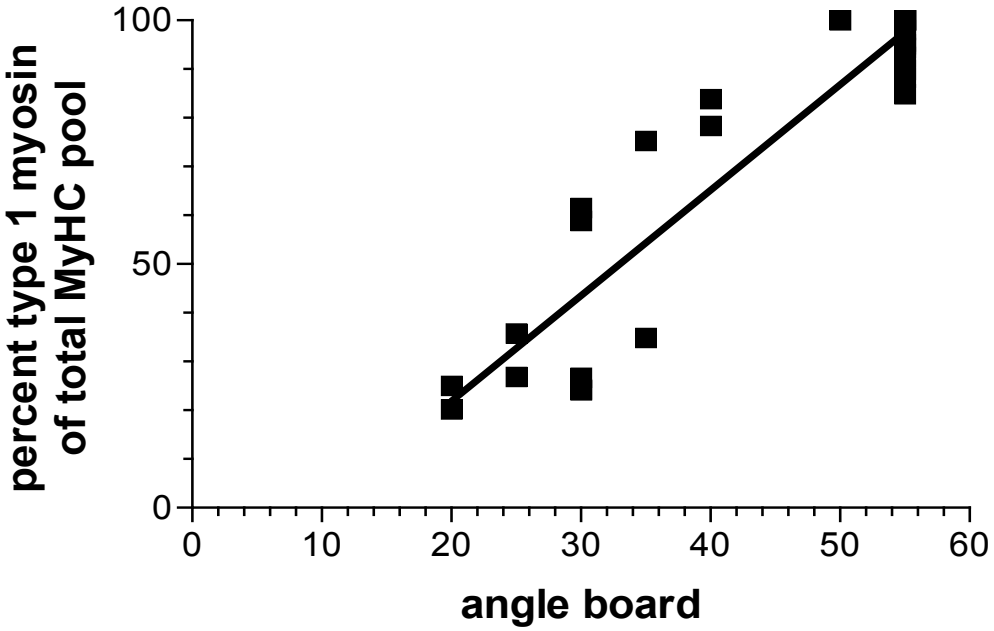

Figure 13. Angle board scores are correlated to the percent of type 1 MyHC in the soleus muscle. Scatter plot displaying percent of type 1 myosin of total MyHC pool from rat soleus muscle and angle board score achieved at six weeks for all rats involved in the study ($p<0.0001$, $r^2=0.8686$).

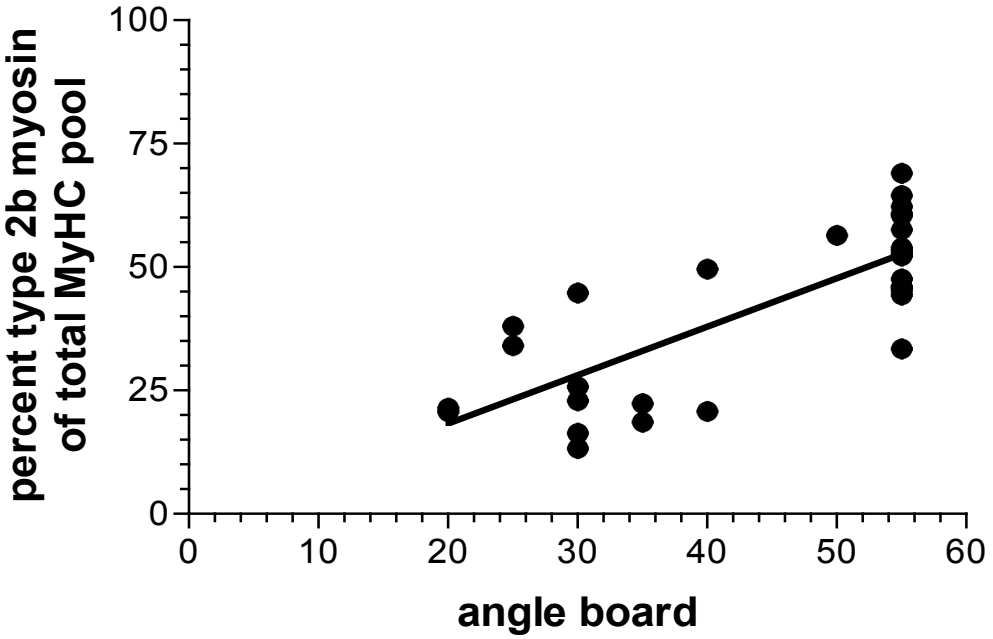

Figure 14. Angle board scores are correlated to percent of type 2b MyHC in the EDL muscle. Scatter plot displaying percent of type 2b myosin of total MyHC pool from rat EDL muscle and angle board scores achieved at six weeks for all rats involved in the study ($p<0.0001$, $r^2 = 0.6084$).

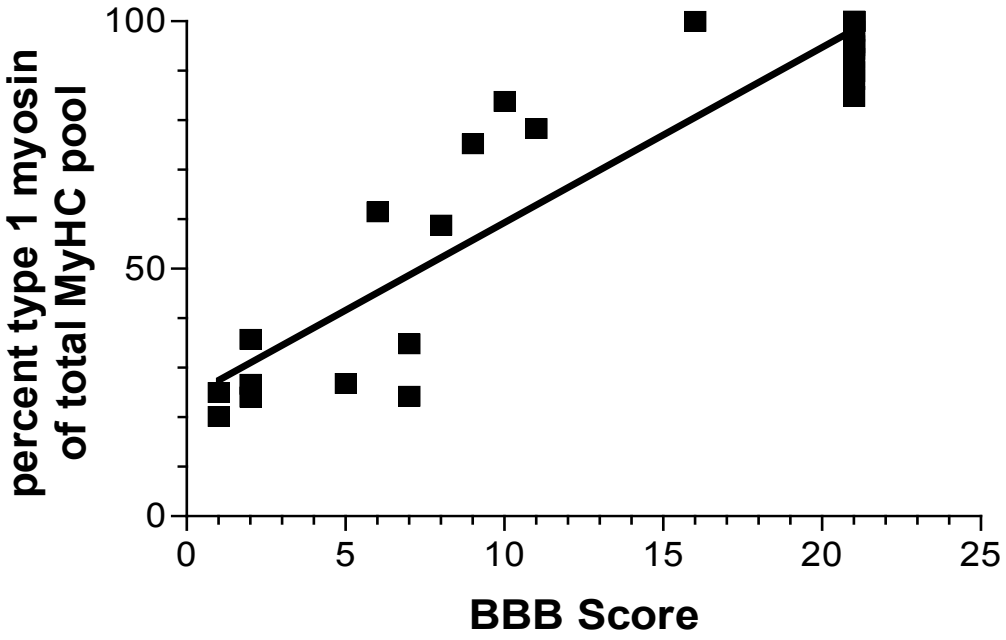

Figure 15. BBB scores are correlated to percent of type 1 MyHC in the soleus muscle. Scatter plot displaying percent of type 1 myosin of total MyHC pool from rat soleus muscle and six week BBB score for all rats involved in the study ($p<0.0001$, $r^2=0.8708$).

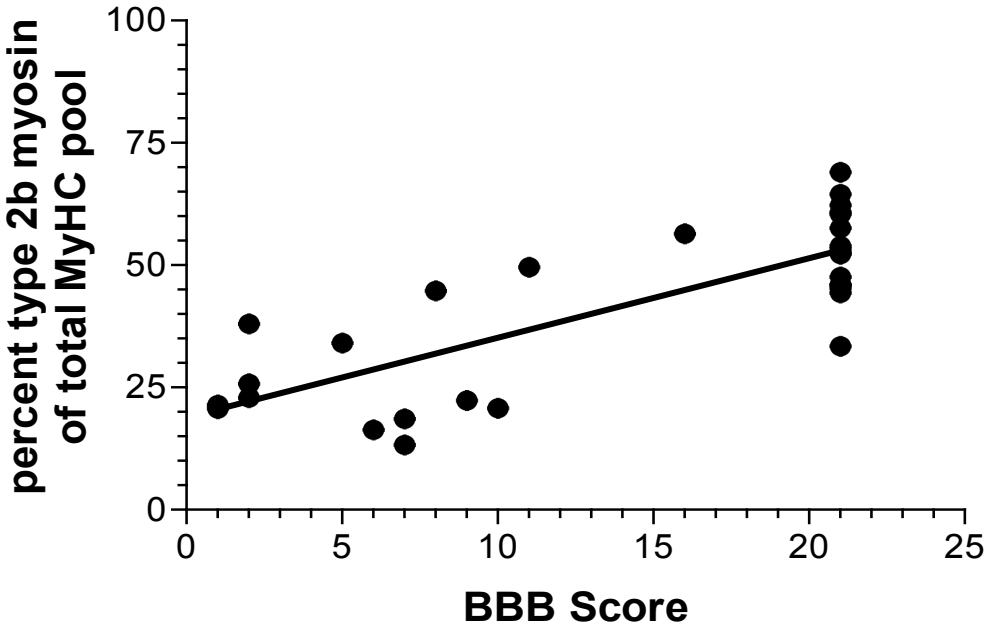

Figure 16. BBB score and percent of type 2b MyHC is correlated in the EDL muscle. Scatter plot displaying percent of type 2b myosin of total MyHC pool from rat EDL muscle and six week BBB score for all rats involved in the study ($p<0.0001$, $r^2=0.6290$).

4. Discussion

4.1 Change in weight

4.1.1 Body weight

Studies have shown that rats with spinal cord injuries have a significantly lower body mass than control rats 15 and 30 days post injury (Talmadge et al., 1999). This is due to the large decrease in weight that SCI rats experience one week after surgery (Huey et al., 2001). The results of this study are in agreement with SCI rats weighing significantly less than healthy and sham rats. However, a significant difference between treated and untreated rats was also noted with untreated rats weighing less than rats from the glutamine treated group.

Glutamine treated rats may be better able to maintain weight compared to untreated rats following SCI. Skeletal muscle is the most important glutamine producer in the body. During states of stress and trauma, protein break down occurs in skeletal muscle in an effort to increase the supply of glutamine to the rest of the body (see Newsholme, 2001; Soeters, 2001; Wilmore, 2001; Calder and Newsholme, 2002; Newsholme et al., 2003). Studies of glutamine-enriched total parenteral nutrition, nutrient rich fluid given by vein, after sepsis have shown increased rates of skeletal muscle protein synthesis and decreased rates of skeletal muscle protein degradation (Ardawi, 1991). Glutamine treatment following SCI would be providing an external source of glutamine to the body, thereby decreasing protein degradation in skeletal muscle.

A second way that glutamine treatment may be helping to maintain weight is by helping to maintain innervation to the muscle. By maintaining innervation, a degree of locomotion is preserved. This is beneficial because if a muscle is not used than it undergoes swift atrophy. This is particularly true for postural muscles that are frequently used under normal circumstances (see Baldwin and Haddad, 2002). For example, the slow soleus muscle is more affected by chronic unloading than fast muscles such as the EDL, plantaris, tibialis anterior, and the gastrocnemius. The soleus shows greater changes not only in muscle atrophy, but also in contractile properties, fiber type composition, and myosin content (see Pette and Starron, 1997).

4.1.2 Muscle weight

Muscle atrophy following SCI is specific for different muscles and varies depending on the kind of muscles. For example, extensor muscles atrophy more than flexor muscles, and slow muscles atrophy more than fast muscles (see Roy et al., 1991; Kraemer et al., 2000).

4.1.2.1 Soleus

It has been shown that the soleus muscle from SCI rats shows significant atrophy (Jakubiec-Puka et al., 1999; Talmadge et al., 1999; Huey et al., 2001). The current study found similar results to those listed above. The soleus muscle, a slow extensor of the ankle (Gutmann et al., 1972; Gupta et al., 1989; Jakubiec-Puka et al., 1990; Esser et al., 1993), from both treated and untreated rats in this study displayed significantly more atrophy compared to healthy and sham rats. This was expected because following SCI rats are paraplegic and have very little, if any, use of their hindlimbs. However,

treatment with glutamine made a significant difference in SCI rats such that the soleus atrophy was lessened compared to untreated rats. As discussed in section 4.2, glutamine treated rats had significantly greater locomotor abilities and hindlimb strength. Increased use of the hindlimbs and hindlimb muscles, particularly postural muscles, may have contributed to decreased atrophy of the soleus muscle.

4.1.2.2 EDL

Unlike the soleus muscle, the EDL did not display any significant differences in weight between any of the groups. While the soleus muscle is a slow twitch ankle extensor, the EDL is a fast twitch ankle flexor and studies of denervation and SCI have shown that the EDL atrophies less than the soleus (Jakubiec-Puka et al., 1990; Jakubiec-Puka et al., 1999; Staron et al., 1999; Hutchinson et al., 2001). In fact, Hutchinson et al. (2001) looked at numerous hindlimb muscles and found that following SCI the EDL was the only muscle that did not experience a significant decrease in muscle weight in the first couple of weeks following surgery. When muscles are unloaded, during chronic bed rest or space flight, the primary targets for protein degradation are muscle fibers containing type 1 MyHC. Notably, the EDL contains very little type 1 MyHCs (Baldwin and Haddad, 2002). Additionally, as stated previously, because the EDL is not a postural muscle it does not atrophy to the same extent as the soleus does with unloading.

Continuous muscle stretch has been shown to prevent muscle atrophy following disuse (Loughna et al., 1986; Roy et al., 1992; Loughna and Morgan, 1999; Sasa et al., 2004). Passive stretch leads to muscle growth by increasing protein synthesis although it does little to decrease protein degradation (Yang et al., 1997). Studies have shown

that passive stretch not only contributes to increased muscle mass, but can also influence MyHC gene expression (Roy et al., 1992; Loughna and Morgan, 1999). Loughna and Morgan (1999) showed that denervation leading to decreased type 1 and type 2a expression in the soleus and the gastrocnemius muscles could be decreased by immobilizing these muscles in a lengthened position following denervation. In another study Loughna et al. (1986) used a suspension model to unload the hindlimbs of rats. While suspended, rats had one leg restrained in a dorsiflexed position with the EDL slack, while the other leg was allowed to hang freely in a plantar flexed position with the EDL stretched. They found that the plantar flexed EDL atrophied to a lesser degree than the EDL in the dorsiflexed position. Passive stretch was effective in reducing muscle atrophy by increasing protein synthesis.

In the present study similar factors may have been responsible for preventing EDL muscle atrophy. After SCI, rats pull their hindlimbs behind themselves with the dorsal part of their foot and toes dragging on the floor. This passively stretches the EDL, but not the soleus muscle. Rats scoring below eight on the BBB scale are not exhibiting plantar placement of the foot. Furthermore, rats with a BBB score below 16 are not displaying any toe clearance during the stepping process (Basso et al., 1995). All SCI untreated rats had BBB scores below 7, and of the SCI treated rats, only one attained a score above 11. Therefore, it is likely that all SCI rats were experiencing stretching of the EDL muscle and this could explain why there were no significant differences in EDL weight among any of the rats.

4.2 Behavioral analysis

4.2.1 Angle board

No significant differences were detected between treated and untreated SCI rats during the first two weeks post surgery. One reason for this may have been due to the large standard deviations seen for the glutamine treated rats. During the first two weeks after SCI there is a lot of variability among angle board scores indicating that rats are recovering hindlimb strength at different rates. Another reason for no significant difference between SCI groups during the first two weeks is that angle board scores were measured starting at a minimum angle of 25 degrees. In retrospect, the minimum angle measured might have been started at a lower angle. This would have ensured that any slight differences between treated and untreated groups during the first two weeks might have been detected. A final explanation could be that at two weeks rats had not recovered enough coordination or strength in their hindlimbs to enable them to maintain their position on the angled board.

After two weeks, treated rats acquired significantly higher angle board scores than untreated rats. However, treated rats never achieved scores as high as healthy or sham rats. This indicated that glutamine administration helps maintain hindlimb strength, but at a reduced level.

4.2.2 BBB scores

Treated SCI rats had significantly better BBB scores than untreated rats at all time points in the study. At six weeks untreated rats achieved a mean score of 2.86, while treated rats had a mean BBB score of 9.57. Comparing the differences in locomotory function of these scores at six weeks highlights the differences between the

two groups. A score of three on the BBB scale indicates that a rat has extensive movement of two of the three hindlimb joints, but is unable to support his weight or take steps. Alternatively, a score of ten indicates that a rat has extensive movement of all three hindlimb joints, can support his weight, and is taking occasional steps. Additionally, the steps are on the plantar surface of the foot rather than on the dorsal surface. However, a score of ten indicates that the rats do not display any forelimb-hindlimb coordination (Basso et al., 1995). In reference to angle board scores, one would not expect that a rat without forelimb-hindlimb coordination would be able to maintain as great of an angle as a healthy rat with perfect locomotory skills.

One point of interest apparent in the BBB scores was that all rats in the SCI treated group obtained the same scores in week five that they did in week six. This may indicate that the treated rats reached a plateau in their locomotory recovery at five weeks. In a study of moderate spinal cord contusion, animals initially experience hindlimb paralysis but regain limited locomotor capabilities with weight bearing within several weeks. Hutchinson et al. (2001) produced by a 1.1mm displacement of one spinal cord surface with respect to the other spinal cord surface using the Ohio State University impact device. The Ohio State University impact device is an electromechanical impactor that is lowered down onto the spinal cord until the desired force is applied (Noyes, 1987). They found that BBB scores of injured rats reached a plateau from three to ten weeks post injury. It has also been found that the Functional Independence Measure score of humans, with a SCI below T6, will plateau at three months post-injury.

Another point of interest becomes apparent when comparing the results that untreated rats attained on the BBB score and the angle board method. Specifically,

untreated rats had different BBB scores at all time points. However, they did not score differently on the angle board during the first two weeks. This difference could be explained as stated previously by not measuring angle less than 25 degrees on the angled board. Nonetheless, this difference in scores between the two techniques may indicate that locomotor ability returns faster than hindlimb strength, or that the BBB score is more sensitive to small changes than the angle board method.

4.3 MyHC isoform expression

It has been shown that muscle atrophy occurring in the first week following SCI is not accompanied by any change in the muscles myosin isoform profile. This implies that during muscle atrophy the absolute amount of myosin is decreasing and has no affect on the relative percentage of different MyHC isoforms. Later however, there is increased expression of some MyHCs and decreased expression of other MyHCs depending on the muscle (Huey et al., 2001). These fiber transitions are evident in the increased expression of hybrid fibers (Pette and Staron, 2000; Talmadge, 2000; Pette, 2001; Pette and Staron, 2001).

4.3.1 Soleus

The soleus muscles of SCI rats displayed a shift in MyHC expression from slow type 1 MyHCs towards faster MyHC isoforms expression. Specifically, in SCI rats there was an increased expression of 2a and 2x MyHC isoforms, but a decreased expression of type 1 MyHC compared to healthy and sham rats. Other studies using various models of decreased neuromuscular activity have found similar results. In a study of spinal cord transection, the soleus muscle from injured rats had an increased expression of type 2a

and 2x MyHCs, and a decreased expression of type 1 MyHCs (Talmadge et al., 1999). Huey et al. (2001) determined that after spinal cord isolation, a procedure producing complete neuromuscular inactivity while maintaining motoneuron-muscle connections, the soleus of injured rats expressed higher amounts of type 2x MyHC and lower amounts of type 1 MyHC. A study of sciatic nerve crush found the same results for the soleus muscle (Jakubiec-Puka et al., 1990).

There were significant differences in MyHC isoform expression found between glutamine treated and untreated rats. Untreated SCI rats had significantly more 2a and 2x MyHC isoforms and significantly less type 1 MyHC isoform expression than glutamine treated rats, which were able to maintain a greater level of type 1 myosin. It is known that intact neural innervation and muscle activity are required for normal energy metabolism, muscle structure and function. For example, changes in neural innervation or muscle activity can increase or decrease metabolic enzyme activity and cause shifts in aerobic-oxidative or anaerobic metabolism. Furthermore, structural changes take place within the muscle causing sarcomeric and membrane protein isoform profiles to be altered. Finally, muscle function is impacted altering contraction and relaxation times (Gupta et al., 1989; Pette, 2001; Pette and Staron, 2001; Pette, 2002). Thus, muscle fibers have the ability to transform and adjust their phenotypic profile to better meet functional requirements (Pette, 2001). Therefore, if glutamine administration following SCI helps to maintain neural innervation and muscle activity, then a more preserved MyHC phenotype would be expected in the soleus muscle of the treated rats.

Despite the differences in MyHC expression among rat groups, there was one similarity for all groups. There were no significant differences in 2b MyHC isoform

expression. This was likely the result of very little 2b MyHC expression in the soleus muscle.

4.3.2 EDL

Contrary to the soleus muscle, treated and untreated groups were not significantly different from one another. However, the EDL of SCI rats was significantly different compared to healthy and sham rats. The EDL from all SCI rats had significantly less type 2a and 2b MyHC isoforms, but more 2x MyHC than the EDL of healthy or sham rats. Type 1 MyHCs accounted for less than five percent of total MyHCs and was unaffected between groups. Thus after SCI, type 2b and some 2a MyHCs were replaced by slower type 2x MyHCs. Other studies have also found that after SCI the EDL displays an increase in 2x MyHC (Hutchinson et al., 2001) and a decrease in type 2b MyHCs (Jakubiec-Puka et al., 1990; Hutchinson et al., 2001).

These results indicate that glutamine treatment alone is not enough to help maintain the phenotypic profile of the EDL. Although glutamine treatment helps to maintain hindlimb strength and locomotor abilities, these effects likely play a more important role in the maintenance of the soleus muscle. During locomotion, slow twitch, postural muscles are more active than phasic muscles such as the EDL (Pette and Vrbova, 1985). Furthermore, even with glutamine treatment, rats have limited recovery and likely do not maintain enough function where fast muscles would benefit because fast twitch muscles fibers are recruited for intense bursts of activity.

4.3.3 Diaphragm

Rat diaphragm myosin was run as a control to visualize MyHC bands in every gel because it displays all four MyHC isoforms. However, the same diaphragm myosin sample was not used for every gel and as a result differing amounts of various myosin isoforms can be visualized in the gels shown in figure 12. The reason for the differing amounts of MyHC isoforms seen in different myosin samples from the diaphragm are due to the fact that the diaphragm has a heterogeneous fiber type distribution (Sugiura et al., 1992). There are significantly more type 1 muscle fibers in the ventral costal region compared to the crural region of the diaphragm. Additionally, the crural region has significantly more 2b fibers compared to the costal diaphragm (Metzger et al., 1985). Finally, in figure 12A there is an additional band below type 1 MyHC in the diaphragm sample. This band is a degradation product. Although muscle samples were stored at -80ºC, myosin will start to degrade over an extended period of time.

4.3.4 Differences in fast and slow muscle regulation

Much research supports differing properties of fast and slow muscles, and how their regulation may differ. Gutmann et al. (1972) was one of the first to note that fast and slow muscles react differently to denervation. The EDL developed a prolonged contraction time, while the contraction time was shortened in the soleus. It was subsequently observed that regulation of the same MyHC isoform may vary in different muscles (Jakubiec-Puka et al., 1999). During development and regeneration, nerves play different roles in fast and slow muscle phenotypic profiles. For example, fast nerves are not necessary for the development of fast muscles, but slow nerves are necessary for the development of slow muscles (see Esser et al., 1993; Huey and Bodine,

1998). Finally, fast and slow muscles are activated differently during locomotion. Slow muscle fibers are used to a much greater extent than are fast fibers. Additionally, while fast muscles are only active during locomotion, slow muscles are active during both locomotion and postural maintenance (Pette and Vrbova, 1985).

4.4 Correlation between behavioral scores and MyHC expression

Angle board scores and BBB scores both had a strong correlation to the amount of type 1 MyHC present in the soleus muscle. The correlation between behavioral scores and the amount of type 2b MyHC present in the EDL was also quite strong although to a lesser extent than those of the soleus. The correlation between higher behavioral scores and a more preserved MyHC profile indicates that improved functional outcome is related to the maintenance of a muscle's myosin profile. This is particularly true for slow twitch muscles.

For both the soleus and the EDL, BBB scores were slightly more correlated to MyHC profile than angle board scores. This may suggest that locomotor recovery is a better indication of muscle preservation than hindlimb strength is. Nonetheless, the strong correlation between behavioral scores and MyHC profile indicates that if MyHC phenotypic profile is being maintained, locomotion is being salvaged as well.

4.5 Hypothesis validation

It was hypothesized that intraperitoneal glutamine administration following SCI would improve functional outcome and hindlimb strength in rats. Additionally, that improved functional outcome would be accompanied with preserved MyHC profile in the soleus and EDL muscles in the rat. The hypothesis was confirmed although MyHC

profile was more affected in the soleus than in the EDL. Histological analyses of the spinal cords from the same group of rats were carried out by Rigley et al. (2004). They demonstrated that SCI rats receiving glutamine treatment had greater white matter sparing than untreated SCI rats (Rigley et al., 2004). Furthermore, they found that intraperitoneal injections of glutamine were effective in increasing blood glutathione levels and maintaining them.

Glutamine administration to SCI rats works by decreasing oxidative stress which increases basal glutathione levels. As a result, there is a decrease in the amount of secondary spinal degeneration following the primary injury and increased tissue sparing. This leads to maintenance of the 'normal' MyHC phenotype, and preserved locomotory function. Additionally, glutamine administration following SCI helps rats maintain body weight and skeletal muscle weight by providing 'conditionally essential' glutamine to the rats, thereby decreasing skeletal muscle protein degradation.

4.6 Future directions

Future studies could be conducted to determine how glutamine is affecting other properties, besides MyHC isoform profiles, of fibers. For example, immunohistochemistry experiments could be carried out to determine if glutamine treatment has any effect on maintaining fiber size and the number of fibers present within a muscle. Additionally, immunohistochemistry experiments could be used to determine the effect of glutamine on the hybrid fiber population of muscles; if there are more or less fibers expressing more than one MyHC isoform, this would determine if muscle phenotype is being preserved on levels besides MyHC expression in glutamine treated rats.

Another avenue that would be interesting to explore is the effect of glutamine administration in skeletal muscle, specifically, whether glutamine concentration is increased within the skeletal muscle of treated rats. If glutamine was being maintained or increased in skeletal muscles there would be stronger evidence that glutamine is helping to preserve muscle by preventing protein breakdown rather than just through maintaining the muscle-neural connection.

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